Enabling Marker-assisted Breeding for Fruit Texture Traits in Progeny of the Apple Cultivar Honeycrisp

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Dedication

This thesis is dedicated to the memory of my grandfather, Thomas Herman (1926-2012) of Clover Valley Farm in Harshaw, WI.

Abstract

Apple fruit texture is a major determinant of consumer acceptability. The cultivar Honeycrisp, a product of the University of Minnesota apple breeding program, is commercially successful and widely recognized for its exceptional fruit texture qualities. Many breeding programs have begun using 'Honeycrisp' as a parent, to develop new cultivars having superb fruit texture and adaptations to unique environmental pressures. This study quantifies fruit texture changes between years and from harvest through storage of fruit from individuals of the three major United States apple breeding programs. A broad range of texture quality was observed within these programs. Using three families with 'Honeycrisp' as a common parent, a 'Honeycrisp' parental linkage map was developed for use in detecting marker-trait-locus associations in the University of Minnesota apple breeding program. Three genomic regions associated with variation for firmness and crispness were identified as regions to target in implementing marker-assisted selection in 'Honeycrisp' descendants.

Table of Contents

List of Tables

List of Figures

CHAPTER ONE: LITERATURE REVIEW

Introduction

Domesticated apple (*Malus* x *domestica* Borkh. Velasco et al., 2010) is one of several fruit crops belonging to the family Rosaceae. Other temperate rosaceous fruit crops include cherry, peach, plum, almond, pear, apricot and strawberry. Fruit breeding in this family has undergone little change over the last half century, but genomics resources and molecular breeding technologies are now available as tools to advance cultivar development (Dirlewanger et al., 2004). Genomic data and analytical tools are hosted at the Genome Database for Rosaceae, a public database (www.rosaceae.org). Like other rosaceous fruit trees, the long juvenile phase of apple, which takes five to ten years to fruiting maturity from seed germination, greatly slows phenotype-based selection of fruit traits. Thus, apple is an ideal candidate for marker-assisted breeding (MAB), which can improve breeding program efficiency by substantially shortening the time between crossing and selection. Other characteristics nominating apple for breeding via molecular tools include high heterozygosity, clonal propagation, self-incompatibility, large plant size, and perennial life history (Luby and Shaw, 2001). Moreover, many apple cultivars today have overlapping pedigrees, increasing the potential for inbreeding depression, associated with loss of vigor (Janick et al., 1996). Pedigree and marker-informed parent selection could greatly increase the probability of accumulating favorable alleles in selected apple seedlings. Breeders making parent choices and seedling selections using genotypic knowledge could release higher quality cultivars at a higher frequency than traditional methods allow.

World apple production reached almost 72 million tonnes in 2009, approximately 6% of which was grown in the United States of America (FAOSTAT, 2009). Consumers recognize apple cultivar names or associated trademarks, in contrast to many fruits such as bananas, blueberries, strawberries, and apricots that are often sold without cultivar distinction. As with most fruit trees, apple cultivar genetic identity is maintained through clonal propagation. Many successful cultivars are the result of open pollinations (e. g., 'Golden Delicious', 'Delicious', 'Granny Smith', 'McIntosh', 'Jonathan') and sports of established cultivars, but modern cultivar releases have their origins in breeding program intentional crosses (Janick et al., 1996). Apple breeding at the University of Minnesota, begun in 1878, focuses on producing cold hardy and, more recently, crisp apples with texture characteristics similar to the popular 'Honeycrisp' cultivar (Luby and Fennell, 2006). Recent assembly of the apple genome sequence by Velasco et al. (2010) will likely change the methodology of this and other apple breeding programs with the availability of inexpensive, high-density molecular marker coverage of the apple genome.

Identifying marker-locus-trait associations to assist breeding for fruit quality traits requires partitioning of the total eating experience into measureable and scalable elements such as aroma, juiciness, hardness, crispness, acidity and sweetness. Sensory panels and instrumental measures help quantify these quantitative traits. Consumers show preference for particular cultivars and quality traits based on their culture, geographic location, familiarity with named cultivars, and childhood exposure (Harker et al., 2003). Complicating the system further, apples of a given cultivar will also vary temporally and spatially in fruit quality. Fruit quality can differ with differences in orchard location and

the associated abiotic factors such as weather and soil type. Two patterns of genotype by environment interaction exist; in the first, individuals rank the same at all locations but differences between individuals are inconsistent across locations, while alternatively with a second pattern of genotype by environment interaction there exist differences in individuals' rank between locations such that the best performer changes with location considered (Lynch and Walsh, 1998). The latter is more challenging for breeders to predict and address. Moreover, changes in fruit texture and taste occur during storage, necessitating repeated measures of fruit quality traits across expected storage duration.

Apple Consumption, Preferences and Texture Perception

The primary target of most apple breeding programs is dessert apples. Dessert apples are fruit that are consumed fresh. Characteristics of interest in dessert apples are outward appearance, eating experience, and the maintenance of such throughout storage (Janick et al., 1996). Processed apples are consumed in various contexts, from baked goods, sauces, jams and juices to dried fruit, and some breeding programs are concerned with traits that influence these products. Self-incompatibility, long breeding cycle, and high heterozygosity in apple make incorporation of superior alleles for each of these traits into one cultivar difficult. DNA marker-assisted breeding (MAB) will help breeders attain this aspiration.

Harker et al. (2003) reviewed studies that investigated consumer preferences in apple and factors influencing willingness to buy. They reported that while segments of consumer populations vary in fruit quality expectations, most adults respond to texture

and acidity as determinants of fruit quality. In a study of New Zealand consumers, adults preferred harder and crisper apples. While Harker et al. (2003) reported that consumers remember differences in apple texture for days, they predicted that fruit quality standards will evolve as consumers' expectations change. A study using 'Red Delicious', 'Gala', and 'Braeburn' showed that, in certain cultivars, firmness is of high importance to consumers; the perceived quality of firm apples (those above a 53 Newton threshold) can be improved upon by changes in titratable acidity (TA) and soluble solids content (SSC), but soft apple acceptance cannot be improved upon with changes in TA or SSC (Harker et al., 2008).

Crispness is highly desirable in dessert apples, but it is important to recognize that researchers do not all use the same definition of 'crispness' in training sensory panelists (see Roudaut et al., 2002 for multiple examples of crispness definitions). Moreover, even firmness and juiciness have been defined differently across studies. Considerations include whether panelists assess the trait during biting or chewing, which part of the tissue they bite, and if they base their assessment on tactile impressions or also consider sounds during biting or chewing. Fillion and Kilcast (2002), using a trained sensory panel and a consumer panel, defined the term 'crunchy' as describing lower pitched sounds that continue throughout chewing while 'crisp' describes a higher pitched sound resulting from the clean split of the first bite. However, in other studies panelists were directed to assess crispness in terms of the "crunchy noise when chewing" (King et al., 2000). Mehinagic et al. (2004) omitted crispness and firmness from sensory attributes and instead measure crunchiness, chewiness, and fondant. Both crisp and crunchy

designations, when applied to food, express that the material breaks in the mouth, rather than buckling or deforming. Studying sounds during biting dry and wet crisp foods, Vickers and Bourne (1976) defined the crispness sensation as a characteristic sound of a range of frequencies emitted during biting. Complicating matters further, perceptions of distinct traits can be altered by other traits; for instance, hardness influences perception of juiciness (Harker et al., 2006). Additionally, consumer perception of sweetness and acidity do not always correlate well with instrumental measures of soluble solids content (SSC) and titratable acidity (TA) respectively (Hoehn et al., 2003).

Quantification of Fruit Texture Traits

Fruit selection and postharvest treatment. Commercially applicable studies of fruit quality traits consider the effects of cultivar, location, harvest time, and, importantly, changes in traits over time under realistic storage conditions. Consequently, fruit texture experiments must be carefully designed to mimic actual commercial treatment of fruits. Considerations such as replication within and across orchard locations, harvest maturity, storage duration, and storage conditions may affect measures of fruit texture changes. Monitoring fruit firmness of 'Royal Gala' and 'Cox's Orange Pippin' in cold storage, Johnston et al. (2002) found an interaction between fruit size and harvest date. Thus, neither fruit size nor harvest date is solely useful in predicting the storability of a given cultivar's fruit. A two-year fruit softening study of 13 replicated cultivars, grown on various rootstocks and of various ages, found significant main effects of cultivar and year but not of replicate trees or cultivar by year interactions (Iwanami et al., 2005). Genotype

was responsible for 57.3% of the observed variance in softening rate, based on measurements of firmness with a pressure probe (Iwanami et al., 2005). That genotype explains the largest proportion of softening variance emphasizes the potential for texture improvement through MAB.

Sensory Evaluation of Texture MAB should lead to the release of cultivars that are more appealing to consumers, especially in terms of eating experience. The first stride to this end is to identify markers that correspond to human perceptions of favorable fruit qualities, necessitating extensive sensory quantification of fruit traits. Conversely, markers for alleles of particularly unacceptable characteristics would aid in eradication of such traits. Sensory studies vary in terms of the level of training panelists receive, panelist familiarity in judging apple traits, nature of responses gathered, definitions of traits for scoring, panel demographics, number of panelists, and degree of replication within the study. Regardless, a trained sensory panel comprising as few as three experienced individuals has been shown to be reliable in a postharvest study (Brookfield et al., 2011). In fact, the panel was able to discern greater separation between cultivars than could be achieved with instrumental measures. With the small panel, Brookfield et al. (2011) found significant effects and interactions between sensory panelist and cultivar for the response variables juiciness and crispness but determined cultivar was the strongest predictor of these trait qualities. While sensory panels are more directly similar to consumer experience and perception of fruit texture, they can be difficult to

standardize, costly, time consuming, and result in panelist fatigue in a breeding program with many hundreds or thousands of apples to evaluate.

Instrumental Evaluation of Texture Sensory panel data are difficult to replace entirely, due to many factors, yet there are obvious incentives to relying on instrumental measures. When instrumental scores can be associated with sensory perception, substituting instrumental measures for human panels could be more accurate in large experiments where panelists can become fatigued. Variations in measures between instruments are more easily controlled, calibrated or otherwise corrected for than those between human panelists. A great diversity of mechanical means exist to quantify fruit texture. Those that are most closely indicative of sensory response are most useful in making selections.

Puncture tests are widely utilized for industry quality checking as well as in fruit texture research (e.g. Costa et al., 2010b; Harker et al., 2006; Kenis et al., 2008; Liebhard et al., 2003). In this type of test, a probe is pushed at a constant speed into the fruit flesh, typically after removal of a thin disc of skin, and force per distance required to progress through the cortex is recorded. Puncture tests, performed with various mechanized penetrometers, are typically used to estimate firmness and juiciness (e.g. Harker et al., 2006). Popular penetrometers include the Magness-Taylor pressure tester and the Effegi fruit-tester (Blanpied et al., 1978). Harker et al. (2002) found puncture tests superior to chewing sounds and tensile measurements in forecasting sensory panelists' perception of texture traits. The Mohr® Digi-Test computerized penetrometer, collecting constant velocity measurements, captures data that correlate well with sensory firmness and

sensory crispness (Evans et al., 2010). This is especially useful, as crispness has proven difficult to measure instrumentally.

Many other instrumental tests have been devised to quantify aspects of fruit texture. These vary in reliability in predicting sensory measures. Some instrumental tests include compression, visible-near-infrared spectrophotometry, cellular imaging of fruit flesh, dry matter content, a variety of acoustic measures, fracture tests, and tensile measurements (Mehinagic et al., 2004; Mann et al., 2005; Palmer et al., 2010; Roudaut et al., 2002; Tong et al., 1999; Zdunek et al., 2010). Compression tests can be an indicator of mealiness and juiciness after storage (Mehinagic et al., 2004). Zdunek et al. (2010) found contact acoustic emission acceptable in predicting sensory crispness. Another texture analyzer, the single-edge notched bend test involves placing a rectangular prism of fruit cortex, which has been notched at the center, between two supports with the notched end down. Pressure is applied above the notch from above with a probe. Brookfield et al. (2011) compared mean measurements from a small sensory panel, an Effegi penetrometer, and two single-edge notched bend devices on 50 to 100 fruit across nine cultivars, and reported that the magnitude of difference between technique scores varied with cultivar. This observation emphasizes that breeders should not depend on a single instrumental trait measure in making texture quality selections, since texture is multifaceted in terms of both fruit physiology and human perception.

Physiology of Fruit Texture and Underlying Genetics

Apple fruit progress through a continuum of complex maturing and ripening processes, reaching harvestable maturity on the tree and ripening past salable quality sometime later when they become unappealing to consumers. Many enzymes are involved in the ripening process. Out of 1,563 complimentary DNA (cDNA) clones from several developmental stages of 'Prima', Soglio et al. (2009) found 285 to be differentially expressed across fruit development. Changes in molecular composition of fruit do not cease after fruit maturation and harvest, however. Key in strengthening economic viability of apple cultivars is lengthening the marketable period before fruit decay. Ideal apple cultivars maintain quality traits such as acidity, sweetness, aroma, and desirable texture through several months of storage. Although the heritability of some fruit quality traits is similar throughout storage, differences were observed in heritability of sensory crispness and firmness during storage (Kouassi et al., 2009). Thus, understanding cellular characteristics influencing harvest cortex texture and texture changes during fruit maturation and storage is useful in developing markers to select dessert apple cultivars with superior texture.

Variability in fruit firmness results from differences in cell-cell packing and adhesion, turgor, cell shape and size, and cell wall fortification (Toivonen and Brummell, 2008). Other texture quality traits such as crispness and juiciness are also linked to variations in cellular morphology. Lin and Pitt (1986) found cell turgor affects type of tissue failure under pressure in apple cortex at several induced turgor pressures, observing plasmoptysis at the highest pressure, cell rupture in intermediate treatments,

and cell de-bonding at the lowest turgor pressures. Recent research also describes three categories of tissue failure (Harker et al., 2002; Allan-Wojtas et al., 2003). Cells may break near the equator in very firm tissues. Alternatively, cell rupture or bursting has been correlated with intermediately firm tissues, while cell-cell de-bonding is associated with very soft tissues that have a mealy mouth-feel. Mann et al. (2005) found significant correlation between cell size and sensory perception of juiciness in apple. Moreover, superior firmness and crispness in 'Honeycrisp' has been attributed to the cultivar's maintenance of cell wall integrity and its high turgor potential (Tong et al., 1999). The many aspects of cellular morphology influencing texture may translate into diverse genetic opportunities for improvements in fresh apple texture.

Apple fruit are perishable, and successful cultivars preferably maintain their unique characteristics through several weeks or months of postharvest storage. Recognized causes of fruit texture changes after harvest include variation in enzymes and other polypeptides regulating ethylene biosynthesis and transduction, cell wall degradation, lignification of cell walls, and expansins (Li et al., 2010). These known mechanisms affect cellular integrity or intercellular cohesion. Ethylene, a plant hormone, plays a significant role in ripening of climacteric fruit such as apple, tomato, peach, and banana in which high concentrations trigger rapid ripening (Li et al., 2010). In apple, Johnston et al. (2001) described three phases of ripening at low storage temperatures: a slow softening, followed by rapid softening paired with increasing internal ethylene concentrations (IEC), preceding another slow softening. Johnston et al. (2002) proposed that the rapid ripening phase is initiated when IECs exceed 1.5 $\mu l\ l^{-1}$ and that the

threshold of 0.1 $\mu l\ l^{-1}$ suggested by others may initiate the first slow softening phase. The ethylene biosynthetic pathway, in which ACC synthase generates 1-aminocyclopropane-1-carboxylic acid (ACC) from S-adenosyl-L-methionine and ACC is converted into ethylene by ACC oxidase (ACO), has received considerable attention from postharvest biologists in the past several years (Zhu and Barritt, 2008). Confirming prior studies relating ethylene concentrations to softening, Defilippi et al. (2005) found antisense suppression of ethylene biosynthesis genes in transgenic apple reduced molecular indications of ripening, which could in part be restored by supplementation with exogenous ethylene. In addition to ethylene-related changes, expansins loosen cell walls during ripening, and depolymerization of pectins and matrix glycans plays a role in fruit ripening and softening (Toivonen and Brummell, 2008). The complexity of ripening mechanisms mirrors the incredible diversity of maturation and ripening habits observable in marketed cultivars today.

Experimentation probing the genetic control of diversity for apple texture traits has met some success. In apple, genes are typically not validated by transformation due to regeneration difficulty and long life cycle, but rather are confirmed through comparison of multiple genotypes and mapping in additional populations. Genes of interest are those associated with fruit texture as it reaches maturity and those associated with ability to maintain firmness and crispness in storage. Genes causing variation for texture at maturity are thought to influence fruit cell-to-cell adhesion, cell size and cell turgidity, while those controlling maintenance of texture through storage are genes involved in the ripening process and degradation.

Several texture genes have identified roles in changes in fruit quality. *MdMADS2.1*, homologous to the *Arabidopsis* fruiting gene *FRUITFULL*, is thought to play a role in apple fruit texture (Cevik et al., 2010). Costa et al. (2008) mapped the expansin gene *Md-Exp7* on LG1 and confirmed cosegregation of softening differences in 31 apple cultivars with three allelic combinations of the gene. Many works identify alleles of *Md-ACO1* (LG10), *Md-ACS1* (LG15), and *Md-ACS3a* in the ethylene biosynthesis pathway, relevant in apple ripening and storability (Costa et al., 2005; Costa et al., 2008; Harada et al., 2000; Wang et al., 2009). Oraguzie et al. (2004) found that after 20 days storage at room temperature, *Md-ACS1-2/2* genotypes were typically significantly firmer than *Md-ACS1-1/1* genotypes of the same harvest seasonality. A similar experiment in cold storage demonstrated again that *Md-ACS1-2/2* allelotypes soften more slowly than the other two *Md-ACS1* alleleotypes (Oraguzie et al., 2007). Zhu and Barritt (2008) found that fruit from genotypes homozygous for both alleles *Md-ACS1-2* and *Md-ACO1-1* had firmer fruit at harvest and after two months of cold storage, with some exceptions. Determining polymorphisms in fruit cell morphology genes and ripening cascade genes precedes the creation of allele-specific molecular markers that can be used for seedling selection and purposeful breeding decisions. Less precise markers (those segregating with the trait, but not necessarily found within the trait-controlling gene) can be more easily developed to select for quantitative trait loci (QTL) alleles associated with increasing fruit quality, without necessitating a complete understanding of the underlying genes.

Apple Genetics and Molecular Markers

Apple is diploid with 17 chromosome pairs. Based on analysis of the 16.9-fold whole-genome shotgun sequence of the estimated 742.3 Mb 'Golden Delicious' genome, Velasco et al. (2010) predicted 57,386 apple genes. The availability of the genome sequence will speed identification of genes of interest and their functional alleles. Genomic sequence data accelerate marker development and enable visualization in genome browsing tools. Quantity and type of markers available play significant roles in the depth and detail at which geneticists can map marker-trait-locus associations and employ them in breeding.

Early genetic studies in apple showed much success with emergent molecular techniques. Genetic maps were developed for cultivars such as 'McIntosh', 'Rome Beauty', 'Prima', 'Fiesta', and 'Discovery' using biparental populations and various marker types including isoenzymes, restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP), and simple sequence repeats (SSR), or combinations of the aforementioned and other markers (Maric et al., 2010). Early mapping populations permitted the identification and placement of genes for disease resistance, self-incompatibility, allergens, fruit traits, and tree form, as well as many other traits and enzymes (Maric et al., 2010).

Currently, the two most informative types of markers used in apple are SSRs and single nucleotide polymorphisms (SNPs). SSRs detect short tandem repeats known as microsatellites that often vary in repetition length between cultivars. Polymerase chain

reaction (PCR) amplification with primers flanking a microsatellite locus gives unique fragment lengths for differing alleles. SSRs thus detected are codominant markers, informative for alleles of both homologous chromosomes, with the exception of the inability to distinguish between homozygotes and hemizygotes since only one common fragment length is determined in both cases. Heterozygotes yield two SSR product lengths, one for each allele. The multi-allelic character of SSRs lends itself to QTL mapping in outbreeding apple (Gianfranceschi et al., 1998). Apple's high heterozygosity, a result of self-incompatibility, favors codominant marker use for QTL mapping, and highly informative SSRs are transferable between cultivars (Liebhard et al., 2002).

SSR markers have been used in apple for both parentage determination and trait mapping. Cabe et al. (2005) used a set of 11 SSR loci to create identifying fingerprints for 'Honeycrisp' and used the marker set in an attempt to resolve the parentage of 'Honeycrisp' by genotyping potential parents identified from University of Minnesota breeding records. Increasingly dense linkage maps resulted from the addition of SSR markers to earlier segregating population maps that were initially based on combinations of isoenzyme, RFLP, RAPD, and AFLP markers (Gardiner et al., 2007; Liebhard et al. 2003). A widely cited linkage map based on the 'Fiesta' × 'Discovery' population, has been continuously bolstered with additional SSR markers, derived from the literature, genomics libraries, EST banks and SSRs from other *Maloideae* species (e.g. Liebhard et al., 2002; Silfverberg-Dilworth et al., 2006).

SNPs occur where DNA variation between alleles is due to a single nucleotide substitution. Prior to having an available genome sequence, apple SNP markers were

found in expressed sequence tag (EST) collections and mapped to apple linkage maps (Chagné et al., 2008). Detecting SNPs in a species with a sequenced reference genome involves shotgun sequencing additional cultivars and aligning the fragments to the reference for comparison. With the sequenced genome, spacing SNP markers with confidence across informative parts of the apple genome became feasible. One shortcoming of detecting SNPs defined based on the reference apple genome sequence is that the marker set is limited to genomic areas present in the 'Golden Delicious' genome (Chagné et al., 2008). Re-sequencing of 'Golden Delicious' and sequencing of additional *M. domestica* individuals will help create a more reliable apple genome map.

Following publication of the genome sequence, the International RosBREED SNP Consortium (IRSC) apple Illumina Infinium® II 8K SNP array v1 was developed (Chagné et al., 2012). SNPs were detected through sequencing of 27 additional apple cultivars, representative of breeding germplasm used the world over. The Apple Infinium® II array v1 consists of 7,867 SNPs in 1,355 clusters (Chagné et al., 2012). SNPs were chosen in clusters of 4 to 10, enabling the identification of functional haplotypes. Determination of SNP genotypes on the Illumina BeadChip array involves hybridization of amplified, then fragmented genomic DNA to array beads carrying oligonucleotides specific to each SNP of the assay. Following hybridization of genomic DNA to SNP-specific oligonucleotide-carrying beads, fluorescently labeled nucleotides are added to the hybridized target, resulting in green or red fluorescence for homozygous samples or a moderate intensity of both red and green for heterozygous samples. Intensity

data at each SNP-specific bead of the array is used, after normalization accounting for differences across several arrays used in an experiment, to determine sample genotypes.

Mapping Marker-Locus-Trait Associations

The history of mapping in major segregating apple populations is presented in Gardiner et al. (2007) and Marić et al. (2010). Linkage mapping in apple has traditionally been done in the F_1 generation using the two-way pseudo-testcross method described by Grattapaglia and Sederoff (1994). The pseudo-testcross method overcomes map construction barriers caused by parental heterozygosity, as no method currently exists to develop homozygous apple individuals. Up to four alleles may be segregating at each marker locus in an apple F_1 progeny - as opposed to F_2 or BC_1 mapping populations, developed in inbreeding plants, in which only two alleles segregate. With the two-way pseudo-testcross method, a map can be developed for each parent using codominant markers segregating for that parent and homozygous for the other parent. The two maps may then be merged. In Joinmap 4.1 (van Ooijen, 2011a), the integrated map is calculated first by averaging parental distances between anchor markers (those segregating in both parental meioses); and then, the integrated map is populated with markers mapped to only one parent, based on their position between flanking anchor markers (van Ooijen, 2011b).

Marker-trait associations detected in biparental populations are not always transferable to other genetic backgrounds. Recently, pedigree genotyping (also called pedigree-based analysis), an alternative to detecting associations in one segregating

population, has earned the interest of fruit breeders. Van de Weg et al. (2006) identify four advantages to pedigree genotyping: plant material exists already in the breeding program, permanence of the study exists within the program across generations, results are directly applicable to the program, and the potential exists to support studies of QTL interactions. An applied example of family-based QTL detection was provided by Rosyara et al. (2009) in detecting Fusarium head blight resistance in wheat.

A unique advantage to breeding in clonally propagated horticultural crops such as fruit trees is that several generations of founding cultivars are typically grown alongside recent selections. Genetic material is not as readily lost as it can be in agricultural crops. This allows apple breeders to retroactively confirm parentage, examine allele flow, and make phenotypic comparisons to older cultivars. Another advantage is that phenotypic data can be gathered over many years on the same individuals for robust characterization, whereas with fewer years of data, trait determination is more environmentally impacted. Clonal retention of founding genotypes additionally lends itself to pedigree genotyping within selected material. Selected material and breeding crosses, available in the breeding program and representative of the program's germplasm, can both be included in one analysis for QTL detection.

Mapping of marker-locus-trait associations, or QTL is in an early phase in apple compared to other crops (Marić et al., 2010). Interaction of multiple genes with the environmental conditions controls many fruit quality traits, complicating detection of robust QTL (Kenis et al., 2008). Even major QTL (responsible for over 20% of the variation) determined in one population will not necessarily be detected for another cross,

at other locations, or even in the same individuals in a different year (Kenis et al., 2008). Accurate positioning of small effect QTL will depend on dense saturation of the genome with markers and standardized phenotyping of several germplasm sets in multiple locations.

Efforts in mapping of QTL in apple for texture traits have utilized an assortment of segregating populations and linkage maps of increasing marker saturation, resulting in diverse and sometimes overlapping conclusions. In progeny of 'Prima' × 'Fiesta' phenotyped at six sites over two years, King et al. (2000) found QTL for a penetrometer firmness measure and sensory data on linkage groups (LG) 1 and 10, and additionally, a QTL on LG16 explaining 17% of the variance in crispness and 30% of the variance in sponginess. King et al. (2000) define crispness in terms of "crunchy noise when chewing", while many reserve the expression to describe an experience during the first bite. In a study of QTL for mechanical texture assays, measuring wedge distance (in which a wedge was driven into fruit cortex tissue until cracking occurred and the crack length was reported), King et al. (2001) found a QTL on LG16 correlating to sensory juiciness and crispness, and they found QTL on LG07 and LG15 for work of fracture and force at crack propagation, respectively. In a progeny of 'Fiesta' × 'Discovery', Liebhard et al. (2003) reported QTL for fruit firmness on LGs 3, 11, 12, and 14, measured with a Magness-Taylor penetrometer over two seasons in three locations. In a population of offspring of the cross 'Telamon' × 'Braeburn', Kenis et al. (2008) consistently found QTL for flesh stiffness on LG16, for firmness on sun side on LG02, and for firmness on shade side on LG10.. A distinction between sides of the fruit exposed to the sun or

shaded from the sun while on the tree is made because fruit typically color more intensely on the hemisphere facing to the outside of the tree, and this can correlate with physiological differences in the fruit cortex. These differences can be accounted for by measuring traits on both sides of the fruit or by taking measures at the interface between the sun and shade sides. Differences in QTL placement between these various studies may be a result of differences in instruments used, differences in texture trait definitions, environmental factors, or the genetic backgrounds of populations in which the studies were conducted.

Kenis et al. (2008) also detected a shift in QTL placement for quality traits between newly harvested and stored fruit samples. Costa et al. (2010b) confirmed QTL presence on LG10 for fruit firmness and softening and determined that *Md-PG1* relates to ripening at room temperature while *Md-ACO1* relates closely to ripening in cold storage. The results of these studies indicate that several areas of the apple genome are significant in determining fruit texture and these areas can contribute variably in accordance with genomic background, experimental methods, and environmental context during fruit development and storage.

Up to this point, only full-sib experimental populations have been considered. An alternative to finding and comparing QTL in several full-sib mapping populations is analysis examining several generations of related populations at once. Bink et al. (2008) describe three advantages of studying inter-related families with multiple founders, as opposed to individuals sharing the same parents: improved ability to detect valuable QTL alleles, relevance of detected QTL in the breeding population, and cost effectiveness. The

cost of genotypic characterization of individuals with select markers is becoming increasingly practical in comparison to the cost of assessing the phenotype the same individuals. FlexQTL™, a software package from Wageningen UR, uses Bayesian analysis with Markov chain Monte Carlo (MCMC) methods to detect QTL with phenotypic trait and genotypic marker data. The European Union High-Quality Disease Resistant Apples for a Sustainable Agriculture (HiDRAS) project was developed as a trial for pedigree genotyping where selections in several related families and progeny are concurrently accessed for QTL (Antofie et al., 2007). FlexQTL™ detects QTL though an iterative process, informed by prior assumptions and guided by the likelihood of the data, resulting in convergence at a posterior distribution of QTL likelihood across a genome, chromosome, or chromosomal region. Bink et al. (2008) demonstrate the utility of FlexQTL™ on a HiDRAS subset population of 604 individuals, comprising 13 full-sib populations, descending from 15 founder cultivars.

Genotype by Environment Interactions

Apple cultivar performance for many traits is widely acknowledged to vary with climate and planting location. In apple, fruit quality traits such as color, shape, overall taste and texture fluctuate across growing locations. Of particular importance to breeders in developing markers for selection is not whether variation occurs with environment, that is, the existence of magnitude changes in trait measures, but whether there are noticeable rank order changes among genotypes when evaluated in different environments. These large rank order changes across environments are one type of

genotype by environment interactions. Biologically significant changes in fruit quality traits could impact consumer satisfaction when experiencing fruit grown in many regions, as apples are available in grocery stores around the year. Miller and Racsko (2012) found that 'Honeycrisp' fruit quality showed substantial variation between grocery stores and purchase times. More research is needed to determine the impact fruit quality variability has on consumer loyalty to apple cultivars. Studies of consumer preference and tolerance for changes in fruit quality traits were discussed in previous sections.

Some studies have attempted to quantify genotype by environment interactions in apple, providing breeders with recommendations on the value of trialing new cultivars in other locations. In open pollinated families of trees evaluated in three sites, Alspach and Oraguzie (2002) determined variance components for crispness, juiciness and firmness, finding significant site by family interactions in almost all instances, but the interaction was always much smaller than the effect of family. A study of twelve genotypes at five locations in Canada concluded that firmness testing at several locations may be sensible, but that for many traits genotype by environment interactions are not large enough as to be economically limiting (Hampson et al., 2009). Hampson et al. (2009) make a case for distinguishing between statistical significance and biological significance, which may be more forgiving. Statistical significance takes into account only mathematical relationship while biological significance would take into account the biological relationships. For instance, while certain genotype by environment interactions may be statistically significant, consumers may not be able to perceive fruit differences across several visits to grocery stores. In a replicated study of 19 cultivars across seven environments, Miller

et al. (2005) found a significant genotype by environment interaction for juiciness, acidity and firmness but not sweetness or crispness. For sensory crispness, planting site and cultivar main effects were significant. The authors recognized that location-specific variation in firmness may be a reflection of fruit size variability by site, but also found significant cultivar by location interactions for instrumentally determined firmness (Miller et al., 2005). Biologically significant genotype by environment interactions deserve attention in traits that are of economic importance, while other statistically significant interactions may be irrelevant in the larger scheme of an apple breeding program. Characterization of consumer perceptions of fruit quality can help make the distinction.

Standardized Phenotyping

An agreed upon, and strictly adhered to, standardized phenotyping protocol across research locations and harvest seasons, both within a species and in closely related crops, can be a powerful tool for breeding advancement in rosaceous fruits. Without high quality phenotypic data, association statistics that link genomic sequence to traits cannot realize full potential (Bassil and Volk, 2010). For successful MAB, the linkages between phenotypic traits and corresponding markers need to be strong. As previously discussed, robust QTL detection is best achieved through observing the species in many diverse growing environments and several years of growth. This requires that all groups supporting a geographically distributed study adhere to a standardized phenotyping protocol. Difficulties in gathering consistent data from a set of breeding programs arise

from differences in vocabulary and reporting methods, measuring protocol and instruments, maturation determination and postharvest treatments (Rudell, 2010). The HiDRAS project accomplished standardized phenotyping in apple across programs in eight European countries, and resulting data were gathered into a repository available to breeders and growers (Gianfranceschi and Soglio, 2004). The apple breeding cohort of the larger RosBREED project based its standardized phenotyping protocol on that of HiDRAS (Evans et al., 2011). Implementing standardized protocol across several breeding programs allows data from those locations to be analyzed together and minimizes confounding of genotype and location effects in the assembled data. Moreover, others may follow the protocol later to supplement the original data, developing a more robust interpretation, or allowing statistically acceptable comparisons between original and additional information when one or more parameters are experimentally changed. Similar traits, such as fruit flesh color, may also be evaluated in closely related crops to find more widely applicable markers and to generate information regarding similarity in genetic control mechanisms.

Marker Assisted Breeding, Marker Assisted Seedling Selection and Marker Assisted Parent Selection

Two ways in which marker-trait association can inform breeding programs for increased efficiency in cultivar development are through marker assisted seedling selection (MASS, also marker assisted selection (MAS)) and marker assisted parent selection (MAPS). In MASS, molecular markers are used to genotype seedlings at loci of

interest, and those with inferior alleles can be segregated or discarded to improve breeding program efficiency. Those progeny reaching maturity are enriched for some characters of interest and selection criteria can focus on other traits. Seedling screening with molecular markers facilitates precise selection for characteristics that may take years to express and could reduce the number of generations for introgression of unique alleles (Dirlewanger et al., 2004). MASS is profitable when heritability is low, the trait is not easily phenotyped in young trees, and when the costs of carrying additional trees to maturity substantially outweighs the cost of marker characterization (Luby and Shaw, 2001).

MAPS, designing crosses using genotypic data to select parents containing marker-loci correlated with the desired trait outcome, ensures favorable probabilities of advantageous allelic combinations in seedling progeny. In comparison to MASS, MAPS only requires genotyping of plausible parent trees, likely substantially less costly than genotypic analysis of an entire progeny. For instance, parents may be chosen based on their breeding value for the trait at the QTL of interest. Both MASS and MAPS, however, require similar initial expense and effort to identify marker-locus-trait associations. Breeders using MASS and MAPS take advantage of molecular tools and genetic knowledge to pyramid desired alleles, achieving a balance between genetic transformation, which can be negatively received by the public, and the traditional, slow, resource-demanding process of traditional breeding.

When molecular markers for selection do not incorporate the functional polymorphism located within the gene of interest, MAS is a type of indirect selection, in

which selection for a specific trait-locus is done by making selections on a correlated locus. Breeders can use indirect phenotypic selection when the trait of interest is difficult to phenotype but moderately to highly correlated with a more easily selectable trait. Sax (1923) recognized that a quantitative trait, bean seed weight, could be indirectly selected upon by way of selection upon a linked morphological marker, bean color, a qualitative trait. This idea extends to molecular markers, in which marker-trait-locus correlations are determined by QTL detection, enabling MAS and MAPS.

MAS can be a powerful tool for breeders, in that it enables selection based on genotype for traits not yet expressed in the phenotype of a plant. For instance, traits expressed only at maturity could be selected upon in juvenile seedlings and traits expressed under environmental stress could be selected upon in optimal growing conditions. A search of literature from 1995 to 1999 returned over 400 articles discussing 'marker-assisted breeding' or 'marker-assisted selection', but with scant mention of actual application of the technique resulting in cultivar releases (Young, 1999). Young (1999) finds poor QTL detection at fault, in that not enough progeny are used and QTL are typically not confirmed over locations, years, and in unrelated populations so as to be universally reliable indicators. Effectiveness of MASS and MAPS is entirely dependent on the recombination frequency between marker and phenotype-conferring genomic sequence, complicating marker development for complex, quantitative traits. Additionally, when locus by environment and locus by locus interactions modify the expressed phenotype, markers identified under one set of experimental conditions may not transfer to alternate environments and genetic backgrounds. In a worldwide survey of

perennial crop breeders conducted in 2007, 14% indicated connection to research involving MAS, and 3% used markers in selection, though not necessarily as the primary determinant of selection (Byrne, 2007). Slow assimilation of MAB in ornamental and fruit crops can be attributed to lagging technology, expense of developing markers, small breeding programs, and high genetic diversity in these more recently domesticated crops, in which introgression of novel alleles receives more attention (Byrne, 2007). Although the potential of MAB in fruit crop development is clear, more extensive and conclusive research is needed to enable its application.

Over the last decade, multiple markers have been identified and offered as tools for MASS and MAPS in apple. Costa et al. (2010a) list the identified ethylene and firmness related genes *Md-ACS1*, *Md-ACO1*, *Md-PG1* and *Md-Exp7* as candidates for MAB, while additionally suggesting that the apple genome sequence will be useful in finding all members of these gene families. Markers for resistance to diseases such as scab and powdery mildew have been developed for apple breeding programs (e.g. Evans and James, 2003 and Hemmat et al., 2002). Recognizing that QTL effect and placement are typically labile over generations within a breeding program, Podlich et al. (2004) propose a "mapping as you go" approach in which QTL are re-determined after each marker-influenced selection, in order to capture genomic-context and lineage-specific shifts in allele value. Effect size, map location, and number of QTL would be expected to change as alleles become fixed, markers are added, and the breeding pedigree is expanded. How breeders choose to utilize marker knowledge in breeding programs may greatly influence MAB effectiveness.

Minnesota Cultivar Honeycrisp

'Honeycrisp', released from the University of Minnesota Agricultural Experiment Station in 1991, has received much attention and increasing commercial importance for its unique "explosively crisp" texture and juiciness and texture maintenance in storage (Greene and Weis, 2001; Mann et al., 2005; Tong et al., 1999). It has since become an important parent in the University of Minnesota's fruit breeding program, as well as in other United States and Canadian breeding programs. 'Honeycrisp', when crossed with another cultivar, offers the potential to produce progeny containing the genetic cause of the texture phenomenon. The other parent can be chosen for other favorable traits not found in 'Honeycrisp' such as disease resistances, tree vigor, and resistance to postharvest disorders. The most firm fruit are *ACO1-1* and *ACS1-2* homozygotes, but 'Honeycrisp' was found to be heterozygous for two firmness genes, *ACS1* and *ACO1,* suggesting its unique texture is not a result of firmness or resulting from the same genetic control as firmness in other successful cultivars (Zhu and Barritt, 2008). 'Honeycrisp' offers a unique texture at harvest, but it also is competitive in that it maintains this texture longer than many other popular cultivars. After 6 months of storage, 'Honeycrisp' apples show less deterioration in the cortex middle lamella than other cultivars (Tong et al., 1999). In principal component analysis of several texture traits, 'Honeycrisp' ranked in the first percentile in a component combining high juiciness with low work to fracture, confirming the uniqueness of this cultivar's texture (McKay et al., 2011). Enabling

marker-assisted selection in progeny of ‘Honeycrisp’ would speed the incorporation of alleles conferring its exceptional texture traits for the development of new cultivars.

CHAPTER TWO: FRUIT TEXTURE TRAITS OF THE RosBREED U.S. APPLE REFERENCE GERMPLASM SET

Introduction

Fruit texture traits, significant to apple breeder decision-making yet unobservable until tree maturity, are ideal candidates for marker-assisted breeding (MAB) and marker-assisted selection (MAS). Marker-locus-trait associations, validated in germplasm relevant to a particular breeding program, facilitate MAB (Bliss, 2010). MAB is used to select parents with favorable alleles and MAS is imposed upon seedling populations to eliminate those with unfavorable allele combinations. Both MAB and MAS can reduce time and expense for new cultivar development in a tree fruit breeding program.

Fruit texture is a focus of breeders because of its role in shaping consumer acceptance of new apple cultivars. Harker et al. (2003) reviewed studies that investigated consumer preferences for apple and factors influencing willingness to buy. They reported that while subsets of consumers vary in fruit quality expectations, most adults respond to texture and acidity as determinants of fruit quality. In a study of New Zealand consumers, adults preferred harder and crisper apples. While the authors reported that consumers remember differences in apple texture for days, Harker et al. (2003) predicted that fruit quality standards will evolve as consumers' expectations change. Speeding the breeding process through the use of molecular markers will aid apple breeders in developing higher quality fruit. A study using 'Red Delicious', 'Gala', and 'Braeburn' showed that in certain cultivars firmness is of high importance to consumers especially in combination with other fruit quality factors: firm apples, above a 53 Newton threshold, can be

improved upon by changes in titratable acidity (TA) and soluble solids content (SSC), but soft apple acceptance cannot be improved upon with changes in TA or SSC (Harker et al., 2008). These findings highlighted the utility of genetic markers to select for fruit texture traits.

Studies of apple texture have used both sensory panels and instrumental measures (e.g., Evans et al., 2010; Ioannides et al., 2007; McKay et al., 2011; and Zdunek et al., 2011). Differences in terms used to describe texture, as well as their definitions, make comparing sensory panel results difficult. For instance, the meaning of the term "crispness" differs across studies. Fillion and Kilcast (2002), using a trained sensory panel and a consumer panel, defined the term "crunchy" as describing lower pitched sounds that continue throughout chewing while "crisp" described a higher pitched sound resulting from the clean split of the first bite. Both crisp and crunchy designations, when applied to food, express that the material breaks in the mouth, rather than buckling or deforming. By studying sounds during biting dry and wet crisp foods, Vickers and Bourne (1976) defined the crispness sensation as a characteristic sound of a range of frequencies emitted during biting. For a thorough discussion of the crispness sensation, refer to Roudaut et al. (2002). In our study, described by Evans et al. (2012), "crispness" refers to the intensity of the cracking noise of the first bite. "Firmness" is equivalent to "hardness" and determined while chewing. "Juiciness" is expressed juice upon chewing. A trained sensory panel, as small as three experienced individuals, has been shown to be reliable in a postharvest study of fruit texture (Brookfield et al., 2011). That panel was able to discern greater separation among cultivars than was achieved with instrumental

measures. While sensory panels more closely mimic consumer perception of fruit texture, they can be time-consuming and difficult to standardize.

Puncture tests, performed with various mechanized penetrometers, are typically used to determine firmness and juiciness (e.g., Harker et al., 2006). Harker et al. (2002) found puncture tests superior to chewing sounds and tensile measurements in forecasting sensory panelists' perception of texture traits. The Mohr® Digi-Test computerized penetrometer captures data that correlate well with sensory firmness and sensory crispness by collecting constant velocity measurements (Evans et al., 2010). This is especially useful, as crispness has proven difficult to measure instrumentally with other devices.

Establishing marker-locus-trait associations for texture traits depends on having an extensive, reliable phenotype database for traits of interest in breeding germplasm. Without high-quality phenotypic data, association statistics that link genomic sequences to traits cannot realize full potential (Bassil and Volk, 2010). Moreover, when standardized phenotyping protocols are used across several breeding programs, the resulting large data sets give more power to studies that detect and characterize quantitative trait loci (QTL) than would be had if each program conducted a smaller, isolated study.

A reference germplasm set of 467 individual genotypes including cultivars, selections, and seedlings was identified as part of the USDA-SCRI RosBREED project. The germplasm set provides efficient allelic representation of current parents in the large, publicly funded U.S. apple breeding programs of Cornell University (CU), Washington

State University (WSU), and the University of Minnesota (UMN). Extensive phenotypic data, including instrumental and sensory measures of fruit texture, were collected on these individuals at each location in the years 2010 and 2011 under three regimes: at harvest, after 10 weeks of cold storage and 1 week at room temperature, and after 20 weeks of cold storage and 1 week at room temperature. Phenotypic data were collected adhering to a standardized protocol (Evans et al., 2012).

The objective in this paper is to elaborate on methods used to obtain data on sensory and instrumental measures of fruit texture traits in the RosBREED apple Crop Reference Set (CRS) and describe variation and repeatability observed for these traits. We also report correlations between sensory and instrumental measures used in this study.

Materials and Methods

Plant material. The RosBREED apple CRS and supplementing individuals included 154 cultivars and parental selections, as well as 313 seedlings of families chosen to provide efficient allelic representation of important breeding parents, for a total of 467 related individuals. Subsets of the RosBREED CRS were grown at the UMN Horticultural Research Center near Chaska, MN, at the WSU Tree Fruit Research & Extension Center in Wenatchee, WA, and at the CU New York State Agricultural Experiment Station in Geneva, NY. Evaluation of a reference germplasm set would ideally include replication of all individuals on the same rootstock at each breeding location. In tree fruits, space and time limitations make such a design impractical. Most individuals, especially seedlings in

breeding families, were not replicated within locations. Due to winter hardiness and other factors, only one (2010) or eight (2011) cultivars were available for evaluation at more than one location. The trees were on several rootstocks and some seedlings were on their own roots. Trees were of various ages as they represented several generations of the breeding programs and different times of propagation. In addition to these potentially confounding factors, different personnel performed fruit quality evaluations at each location.

Phenotyping protocol. The complete RosBREED phenotyping protocol for apple is available at http://www.rosbreed.org/sites/www.rosbreed.org/files/RosBREED.2010-Phenotyping_protocol.Malus_.pdf, and in Evans et al. (2012). Portions of the protocol pertaining to fruit texture measurements are summarized below.

Crop load and fruit harvest. As the amount of crop relative to tree size is known to affect some fruit quality traits (Stopar et al., 2002), crop load was managed so that young trees (3^{rd} to 6^{th} leaf) were thinned to 20-25 fruit per tree, while fruit on mature trees were thinned to four fruit per foot of branch length.

Fruit were monitored near the onset of the harvest season, and weekly inspection determined the most suitable harvest time for each individual (genotype) so that all fruit were assessed at similar maturity. Maturity determination was based on a destructive starch-iodine reaction, described by Blanpied and Silsby (1992), and all fruit to be evaluated were harvested when the representative fruit tested in the orchard scored three

or higher on their scale of 1 to 8. Due to variability across seasons and genotypes affecting starch accumulation and conversion, starch-iodine testing is an imperfect determinant of fruit maturity. When available, up to 20 sound fruit were harvested from one tree to obtain the 15 needed for evaluation. Extra fruit allowed for loss in storage. Fruits that were visibly damaged (e.g., sunburned, cracked, or rotting) or differing substantially from the average in size or maturity (e.g., fruit from the shaded tree center) were avoided. When 15 fruit were not available for harvest, all available sound fruit were harvested.

Up to five fruit of each individual were evaluated at three points in time: harvest; 11 weeks postharvest in which the first 10 weeks were in cold storage and the last week was at room temperature (referred to as 10-week storage); and 21 weeks postharvest in which the first 20 weeks were in cold storage and the last week was at room temperature (referred to as 20-week storage). When less than 15 fruit were harvested, available fruit were evaluated at each time point until no more remained. For instance, if 10 fruit were available for an individual the harvest and 10 week evaluations were made and the 20 week evaluation was omitted for lack of fruit.

Cold storage was at 1 ± 2 °C in normal atmospheric conditions. Fruit were equilibrated to room temperature for 1 day before sensory and instrumental measures, when harvest evaluations could not be made the day of harvest. For storage evaluations, fruit were left at room temperature 1 week prior to evaluation.

Instrumental evaluation. All five fruit were marked to indicate sun-shade intermediate sides by drawing a line with a felt-tipped marker around the fruit where sun and shade skin coloring met. Before penetrometer assessment, a small disc of fruit peel was removed with a mandoline or knife at the apple equator on the marked line on either side of the apple. Only one side of each fruit was probed at the sun-shade interface, except at CU where both sides were probed with an Effegi penetrometer. Side selection was arbitrary at UMN and WSU, but bruised areas and other damage that may cause tissue softening were avoided. Fruit diameter, flesh firmness, and flesh crispness were measured (Evans et al., 2010) with a Mohr® Digi-Test (MDT-1; Mohr and Associates, Richland, WA) penetrometer on factory settings at UMN and WSU. At CU, no instrumental crispness measure was available and fruit diameter was measured with a caliper.

The MDT-1 penetrometer collects data on several fruit texture parameters of interest. Traveling at a constant velocity, the penetrometer measures force required to push a plunger through the fruit flesh over two regions (Figure 2.1). Region 1 is from point of entry to a depth of approximately 8.1 mm, representing the fruit cortex below the skin probed by many penetrometers (Mohr and Mohr, 2000). Region 2, the main edible portion of the fruit, extends from the inside of boundary of region 1 to the core. Force measures were recorded for the average, maximum and endpoint values of the two zones (Ax, Mx, and Ex, respectively). M1 (maximum force in region 1) is comparable to traditional industry penetrometer firmness determinants, and crispness (Cn) is quantified as released energy as the probe advances (Mohr and Mohr, 2000). The quality factor (QF)

is a weighted sum of several MDT-1 traits, where high values suggest high fruit quality (Mohr and Mohr, 2000).

Sensory evaluation. Two trained sensory panelists at each location evaluated halved apple quarters of up to two fruit each for each individual tree when sufficient fruit were available. Slices for sensory evaluation were taken from the intermediate side, directly opposite the penetrometer site of insertion at UMN and WSU. At CU, sensory panelists were given the sun-side portion of the fruit. Firmness, crispness, and juiciness were rated on a 1 to 5 scale. For firmness, 1 = very soft, 2 = soft, 3 = medium, 4 = firm, and 5 = very firm. Crispness was rated from 1 = no noise to 5 = very noisy. The juiciness scale was 1 = dry, 2 = slightly juicy, 3 = medium juicy, 4 = juicy, and 5 = very juicy. Sensory anchors for each trait were described by Evans et al. (2012); for instance, anchors for juiciness were the examples of banana and watermelon, scored 1 and 5 respectively. Panelists provided a single rating of each sensory parameter for each fruit sampled. Panelists held the peel of the apple slice and bit through fruit cortex to determine crispness and chewed for firmness and juiciness assays. A second slice of the same apple quarter was available for confirmation. When fewer than four fruit were available, fruit were divided between panelists. Using the five point scale for each sensory trait, a single score for each individual in the germplasm set was reported in each year it was evaluated at a location. At UMN, the mean scores of the four fruit, two by each panelist, were reported, and at CU and WSU panelists discussed their ratings and reported a consensus score. CU and WSU panelists evaluated one or more fruit each until a consensus score was verbally

agreed upon. Changes in sensory traits were calculated for each individual as the difference between ratings for a trait at harvest evaluation and after storage (e.g., 10-week storage rating minus the harvest rating).

Statistical analyses. Harvest texture traits from individuals having more than one fruit in both seasons were analyzed by analysis of variance (ANOVA) with years and individuals as crossed factors. Years were treated as random effects and individuals were treated as fixed effects. Values for each individual fruit were used in the ANOVA to determine fruit sampling contribution to variance in the diverse texture measurements. Up to five fruit were sampled for each instrumental trait, and four fruit were sampled for each sensory trait. Data for 46 individuals evaluated in both years were available from UMN, of which 17 individuals were seedlings, 22 were selections and seven were cultivars [‘Akane’, ‘Arlet’, ‘Gingergold’, ‘Honeycrisp’, ‘Sawa’, Snowsweet® (cultivar ‘Wildung’), and Zestar!® (cultivar ‘Minnewashta’)]. Data for 105 individuals, comprising 69 seedlings, 17 selections, and 19 cultivars [‘Ambrosia’, ‘8S6923’ (Aurora Golden Gala™), ‘Cameo’, ‘Co-op 39’ (Crimson Crisp™), ‘Cripps Pink’ (Pink Lady®), ‘Delblush’, ‘Delorgue’, ‘Enterprise’, ‘Goldrush’, ‘Hatsuaki’, ‘SPA440’ (Nicola™), ‘Pinova’(Piñata®, Corail™), ‘Scifresh’ (Jazz™), ‘Scired’(Pacific Queen™), ‘Sciros’(Pacific Rose™), ‘Silken’, ‘Nevson’ (Sonya™), ‘Co-op 29’ (Sundance™), and ‘Cripps Red’ (Sundowner®)], were available from both years at WSU. In both years at CU, data for 36 individuals – 32 seedlings, 1 selection, and 3 cultivars (‘Hudson’, ‘Russian Seedling’, and ‘SunCrisp’) – were available. The proportions of total variance accounted for by year, individual,

year×individual interaction, and variation among fruit from an individual were determined for instrumental measurements at all locations and for sensory evaluations at UMN.

For all other statistical analyses, means of the five fruit were used to represent each individual at a location, year, and storage duration. Ranges, means, and standard errors were determined for instrumental and sensory texture traits for each location-year-storage duration instance. As individuals, sensory panelists, and environmental factors within location effects were confounded, means separation would not be informative. Spearman's rank order correlations were determined between non-parametric sensory measures and between instrumental and sensory measures at each location-year-storage instance. Year-to-year repeatability of sensory and instrumental texture measures at each location-storage treatment were estimated using Pearson's product-moment correlation. All statistical analyses were conducted using R (R Development Core Team, 2011) and bean plot graphs were constructed using the beanplot package (Kampstra, 2008) in R. Bean plots for each year included data from individuals assessed in both years and individuals assessed in only 2010 or 2011.

Results and Discussion

In 2010 and 2011, fruit from 216 and 330 individuals, respectively, were harvested and a total of 369 individuals of the CRS were evaluated over the two years. During the 2010 harvest, 73, 90, and 51 individuals were evaluated at UMN, WSU, and CU, respectively, for sensory texture. In 2011, 176, 98, and 56 individuals were evaluated

at UMN, WSU, and CU, respectively. Postharvest attrition of an individual's fruit during storage varied with location and year (Table 2.1). Attrition reflected fruit availability and/or storage potential of the individual in some instances. Frost injury at bloom in 2010 reduced the number of fruit available at CU. A 2010 summer hailstorm likely caused the high attrition from harvest to first storage evaluation at UMN, where fruit samples were limited in availability.

Means, ranges, and phenotypic variances of all individuals at a location differed among locations for some traits (Table 2.2), reflecting the largely unique set of individuals and differing environmental conditions at each location. Average sensory scores diverged little among locations. The majority of texture trait scores and measures decreased in magnitude with increasing storage duration. Higher sensory scores were observed at UMN and WSU at harvest and after the 10-week storage duration than for the 20-week storage treatment. MDT-1 instrumental measures were more acutely different between WSU and UMN means in 2011 than in 2010. This result could be due to marked differences in environmental conditions those years or a reflection of the specific individual genotypes available in each harvest year. Observed instrumental trait ranges were broader for many MDT-1 measures at UMN compared to WSU in 2010.

The individuals considered exhibited a wide range of fruit firmness. Average instrumental firmness (MDT-1 M1 and Effegi penetrometer) of a five-fruit sample representing an individual ranged from 19.6 to 149.4 N at harvest, 9.4 to 124.4 N after 10 weeks cold storage, and 3.3 to 129.6 N after 20 weeks cold storage. Averaging harvest and two-month storage treatment measures, Evans et al. (2010) reported M1 values

ranging from about 40 to 100 N for cultivars including ‘Sciearly’ (Pacific Beauty™), ‘Braeburn’, ‘Cripps Pink’, ‘Fuji’, ‘Honeycrisp’, and others. Using Magness-Taylor, Effegi, and other penetrometers, under treatments varying from three to fourteen months of storage, DeLong et al. (2000) reported firmness ranges from 35 to 75 N for the cultivars ‘Cortland’, ‘McIntosh’, and ‘Northern Spy’. Higher and especially lower values than those reported for commercial cultivars were expected in the apple CRS that was two-thirds constituted of unselected breeding germplasm.

ANOVA indicated that the proportion of variance attributable to fruit sampling, individual, year, and year×individual depended on location and trait of interest (Figure 2.2). The effect of year was a much lesser contributor to texture variation at WSU than at CU and UMN.

Repeatability of sensory texture traits was generally low to moderate, with Pearson’s correlation coefficients comparing 2010 and 2011 data ranging from 0.13 to 0.78 for sensory crispness (WSU at harvest and UMN at 20-week storage, respectively) and 0.30 to 0.81 for sensory firmness (CU at harvest and UMN at 20-week storage, respectively; Table 2.3). Pearson’s correlation coefficients ranged from 0.19 to 0.70 for sensory juiciness (CU at harvest and CU at 20-week storage, respectively). Year-to-year repeatability tended to be higher for instrumental measures than sensory measures (Table 2.3). Year-to-year correlations at WSU and UMN, as measured by the MDT-1 penetrometer, were moderate, with statistically significant correlations ranging from 0.37 to 0.93. Between-year correlations in instrumental firmness, as measured with the Effegi

penetrometer at CU, were 0.70, 0.71, and 0.73, chronologically from harvest through storage.

High trait repeatability across years indicates low genotype × year interaction relative to variation among individuals for a trait. Therefore, response to selection on highly repeatable traits with MAS is expected to be larger than phenotypic selection without markers, even when marker selection is based on associations observed from only a few years of data. While markers for quantitative traits that are relatively stable across years in their expression will be easier to develop and validate, markers for traits affected more by yearly variation may be of higher utility than stable markers, as selection based on phenotype alone will be less reliable.

Correlations across years between sensory and instrumental traits indicated consistent effects of factors affecting these relationships (Table 2.4). In some instances, correlations between sensory and instrumental texture measures were high, especially after 20 weeks of storage. For instance, the MDT-1 A2 measure showed correlations to sensory measures ranging from 0.42 to 0.87 after 20 weeks of storage. The MDT-1 M1 correlated well with sensory firmness. Spearman's rank correlations for MDT-1 M1 and sensory firmness measurements at harvest at UMN were 0.73 and 0.75 in 2010 and 2011, respectively. Evans et al. (2010) observed a correlation of 0.66 between M1 and sensory hardness in fruit from central Washington. With respect to sensory traits, crispness was moderately correlated with firmness (ranging from 0.33 to 0.61 at harvest) and with juiciness (0.35 to 0.70 at harvest), but juiciness was poorly correlated with firmness (0.04 to 0.28 at harvest). Differences in individuals and sensory panelists likely account for

some observed differences in strength of correlations among locations, such as the contrast between harvest MDT-1 M1 to crispness correlations between WSU and UMN (Table 2.4).

The MDT-1 measures A1, M1, and QF were especially predictive of sensory firmness (Table 2.4). MDT-1 C0 was strongly and negatively correlated with firmness (Table 2.4). The utility of the MDT-1 at predicting sensory measures appears to be location-dependent at harvest, as WSU measurements did not show nearly as strong correlations as UMN data. The average sensory to instrumental correlation at UMN was 0.49, while the average at WSU was 0.29. The MDT-1 Cn, proposed to measure crispness, was similarly correlated with sensory firmness and sensory crispness in this study (average correlations of 0.40 and 0.43, respectively, across locations and years). Therefore, the choice to replace sensory evaluation in breeding with a MDT-1 penetrometer may not be recommended for all locations or selection purposes.

Changes in sensory and instrumental trait values from harvest to 10 weeks storage and from harvest to 20 weeks storage were compared visually (Figures 2.3 and 2.4). Statistical comparisons would be complex and not be very informative as few individuals were common across locations as well as years. Additionally, not all individuals evaluated at harvest had sufficient fruit to be analyzed after storage. As expected, larger changes in texture occurred after 20 weeks of storage than after 10 weeks of storage. An exception was 2010 instrumental firmness at CU where the average firmness loss from harvest to 10 weeks storage was 24 N but loss from harvest to 20 weeks storage was 21 N (bold, black bars mark distribution means, Figure 2.4). Rapidly

softening fruit representing some individuals may not have been available at the last evaluation, producing this result. Completely decayed fruit were discarded as they were unsuitable for sensory panelist consumption. Differences in distributions among locations demonstrate that although a standardized protocol with a common scale and anchors was agreed upon, sensory scores represent a more subjective and less repeatable evaluation system than instrumental evaluation. Standardized protocols greatly increase the utility of sensory panel evaluation. On average, sensory scores dropped less than 1 point on the 1 to 5 scale after storage from harvest to 20 weeks, while instrumental measures detected average firmness losses of 24% to 34% after 20 weeks of storage. The relative changes after 20 weeks of storage in sensory and instrumental crispness scores were similar (Table 2.2, distributions not presented) with sensory scores declining 18% to 28% and Cn declining 21% to 46% at WSU and UMN, respectively. In the majority of instances, sensory evaluation detected a proportionally smaller texture loss than that detected by instrumental evaluation.

When using these data for QTL discovery, it is important to consider that harvest sensory scores are not directly comparable to sensory scores later in the experiment. The percent loss in texture measured instrumentally was in most instances greater than that perceived by panelists. Differences in texture distributions between years within a location were primarily differences of distribution shape. Distribution shape differences between years at the same location could be due to different sets of individuals evaluated between the years, environmental effects, or a change in scoring regimen (e.g., WSU

panelists agreed upon a single, integer score in 2011 rather than verbally averaging scores as in 2010).

Conclusions

This paper presents information on fruit texture trait distributions in a reference germplasm set of three breeding programs that can be used to simultaneously detect and validate QTL. The wide ranges of observed phenotypic values for these traits are a prerequisite for detecting marker-trait-locus associations. Due to the lack of replication of many individuals across sites, these data should not be directly pooled for QTL detection, as confounded sources of variation at a location (orchard environment, instruments, and sensory panelists) have not yet been accounted for in this study. QTL detected at multiple locations, through independent analyses of each location, would be of particular interest due to robustness across the confounded factors. The data may be adjusted for confounding factors using relatedness among individuals among locations, resulting in a larger population for examining location effects and for pedigree-based QTL analysis. Knowledge of QTL×environment interactions, especially rank-order shifts in which a QTL effect is ranked of higher utility in one environment than in others, will be pertinent to deployment of MAB and MAS that targets apple production environments beyond those of the three research facilities.

Marked differences among locations in trait ranges for instrumental texture measures may affect the detection of QTL or their effect magnitudes at each location. Germplasm fixed or nearly fixed for extreme-effect alleles at a QTL will have less

phenotypic variation for the trait, perhaps leading to the discovery of lesser-effect QTL but also limiting the ability to detect the large-effect QTL. When predictive markers are developed for application outside the initial germplasm studied, ascertainment bias may limit marker utility, as not all alleles of utility may be present in the initial study. The analysis of phenotypic variation in these three diverse breeding programs across years can help with interpretation of QTL analyses that will follow. Moreover, an understanding of trait variability across years is essential in recommending markers for MAB.

Other researchers may use RosBREED's standardized phenotyping protocols on additional germplasm or years to supplement the original dataset to develop a more robust interpretation or to examine the effects of changing one or more parameters. Individuals in this set have been genotyped with the International RosBREED SNP Consortium (IRSC) 8K SNP array developed by Chagné et al. (2012). Phenotypic and genotypic data for this reference germplasm will be curated and available for use by the international community of apple breeders and allied scientists for QTL mapping and validation as well as other analyses through the Breeders Toolbox application at the Genome Database for Rosaceae (www.rosaceae.org).

As the phenotyping protocol used here is similar to that of the European High-quality Disease Resistant Apples for Sustainable Agriculture project (Evans et al., 2012), a meta-analysis could be very informative. Similar fruit texture parameters may also be evaluated in closely related crops to better understand the traits, to find widely predictive markers, or to generate information about synteny among the respective genomes.

Table 2.1. Counts of individuals phenotyped by year, location, and storage duration for sensory and instrumental traits at the University of Minnesota (UMN), Washington State University (WSU) and Cornell University (CU).

	2010			2011		
	Harvest	**10-week storage**	**20-week storage**	**Harvest**	**10-week storage**	**20-week storage**
Sensory						
UMN	73	34	23	176	144	135
WSU	90	83	79	98	93	87
CU	51	37	35	56	54	50
Total	214	154	137	330	291	272
Instrumental						
UMN	76	35	25	171	146	137
WSU	91	83	79	98	91	87
CU	49	36	33	56	55	50
Total	216	154	137	325	292	274

Table 2.2. Instrumental and sensory texture (firmness, crispness, juiciness) trait means ± standard errors and trait range (minimum, maximum) in 2010 and 2011 at Cornell University (CU), University of Minnesota (UMN), and Washington State University (WSU) at harvest and after 10 or 20 weeks refrigerated storage followed by 1 week at room temperature. See text and Figure 2.1 for description of instrumental measures using Mohr Digi-Test (A1, A2, C0, Cn, E2, M1, M2, OAH, OMH, QF) and Effegi penetrometers.

	2010					
Trait[z]	**CU**		**UMN**		**WSU**	
Harvest	**mean ±se**	**(min, max)**	**mean ±se**	**(min, max)**	**mean ±se**	**(min, max)**
A1	NA	NA	44.93 ±1.39	(9.24, 85.85)	49.08 ±0.67	(32.56, 65.41)
A2	NA	NA	98.89 ±2.43	(29.41, 143.33)	96.04 ±1.14	(68.76, 123.94)
C0	NA	NA	0.12 ±0.08	(0, 5.86)	0.01 ±0.002	(0, 0.10)
Cn	NA	NA	431.32 ±21.67	(10.84, 775.96)	246.21 ±6.8	(60.22, 401.07)
E2	NA	NA	131.73 ±4.16	(31.03, 176.73)	114.82 ±1.63	(81.23, 167.12)
Effegi	73.22 ±2.72	(38.25, 117.43)	NA	NA	NA	NA
M1	NA	NA	73.87 ±2.28	(19.57, 149.38)	78.76 ±1.34	(50.88, 127.90)
M2	NA	NA	140.73 ±3.95	(34.07, 179.63)	121.38 ±1.54	(93.84, 170.11)
OAH	NA	NA	83.32 ±2.12	(24.72, 123.68)	80.22 ±0.93	(58.78, 101.88)
OMH	NA	NA	140.52 ±3.98	(34.07, 179.63)	121.38 ±1.54	(93.84, 170.11)
QF	NA	NA	119.94 ±8.87	(-145.58, 242.95)	113.39 ±3.48	(23.54, 221.97)
Firmness	2.78 ±0.13	(1, 4)	2.91 ±0.10	(1, 4.75)	2.96 ±0.05	(2, 4)
Crispness	2.71 ±0.13	(1, 4)	2.81 ±0.10	(1, 4.25)	2.97 ±0.04	(2, 4)
Juiciness	2.39 ±0.11	(1, 4)	2.93 ±0.09	(1, 4.25)	2.79 ±0.06	(1.5, 4)

Table 2.2 continued (2 of 6)

	2011					
Trait[z]	**CU**		**UMN**		**WSU**	
Harvest	**mean ±se**	**(min, max)**	**mean ±se**	**(min, max)**	**mean ±se**	**(min, max)**
A1	NA	NA	44.34 ±0.74	(19.36, 75.85)	51.53 ±0.71	(33.23, 70.75)
A2	NA	NA	78.8 ±1.59	(8.81, 119.40)	95.82 ±1.14	(71.35, 123.28)
C0	NA	NA	0.71 ±0.15	(0, 9.73)	0.013 ±0.002	(0, 0.091)
Cn	NA	NA	123.13 ±6.12	(0.69, 418.43)	254.47 ±7.4	(100.30, 462.80)
E2	NA	NA	96.31 ±1.72	(43.77, 141.09)	115.42 ±1.63	(80.83, 158.60)
Effegi	79.68 ±2.17	(38.7, 123.44)	NA	NA	NA	NA
M1	NA	NA	73.00 ±1.21	(31.70, 123.26)	80.63 ±1.18	(53.40, 115.09)
M2	NA	NA	96.52 ±2.07	(9.57, 143.41)	120.89 ±1.55	(89.94, 160.66)
OAH	NA	NA	65.34 ±1.12	(33.67, 101.42)	80.87 ±0.95	(60.52, 104.77)
OMH	NA	NA	99.35 ±1.72	(49.54, 143.41)	120.94 ±1.55	(89.94, 160.66)
QF	NA	NA	56.69 ±4.69	(-141.39, 180.63)	116.39 ±3.42	(33.79, 203.38)
Firmness	2.57 ±0.09	(1, 4)	2.92 ±0.06	(1, 5)	2.94 ±0.05	(2, 4)
Crispness	2.31 ±0.08	(1, 4)	2.79 ±0.06	(1, 4.5)	2.92 ±0.06	(2, 4)
Juiciness	2.38 ±0.09	(1, 4)	2.85 ±0.05	(1, 4.67)	2.77 ±0.06	(1, 4)

Table 2.2 continued (3 of 6)

	2010					
Trait[z]	**CU**		**UMN**		**WSU**	
10-week storage						
A1	NA	NA	35.10 ±2.05	(10.17, 71.33)	43.07 ±0.77	(23.84, 59.49)
A2	NA	NA	70.94 ±3.28	(35.07, 105.04)	80.12 ±1.42	(44.31, 112.91)
C0	NA	NA	0.12 ±0.03	(0, 0.97)	0.03 ±0.005	(0, 0.22)
Cn	NA	NA	308.75 ±20.70	(88.58, 528.68)	200.09 ±7.81	(64.29, 398.60)
E2	NA	NA	90.23 ±4.98	(41.21, 155.55)	93.80 ±1.85	(37.51, 144.76)
Effegi	53.14 ±3.91	(13.34, 91.41)	NA	NA	NA	NA
M1	NA	NA	60.82 ±3.65	(22.80, 123.02)	67.92 ±1.34	(37.13, 95.73)
M2	NA	NA	95.96 ±4.97	(47.55, 164.47)	98.88 ±1.83	(51.77, 146.88)
OAH	NA	NA	61.67 ±3.16	(31.43, 109.95)	67.49 ±1.20	(38.90, 93.54)
OMH	NA	NA	97.74 ±5.08	(47.55, 164.47)	98.88 ±1.83	(51.77, 146.88)
QF	NA	NA	42.26 ±15.81	(-150.46, 199.00)	65.36 ±5.19	(-91.16, 174.82)
Firmness	2.49 ±0.15	(1, 4)	2.77 ±0.19	(1, 5)	2.64 ±0.06	(1, 3.5)
Crispness	2.23 ±0.14	(1, 4)	2.38 ±0.16	(1, 4)	2.43 ±0.06	(1.5, 3.5)
Juiciness	2.11 ±0.13	(1, 3)	2.71 ±0.11	(1.75, 4)	2.48 ±0.06	(1, 3.5)

Table 2.2 continued (4 of 6)

	2011					
Trait[z]	**CU**		**UMN**		**WSU**	
10-week storage						
A1	NA	NA	29.22 ±0.89	(5.06, 58.86)	44.13 ±0.81	(25, 69.51)
A2	NA	NA	50.89 ±1.64	(5.478, 98.01)	80.33 ±1.39	(50.8, 125.59)
C0	NA	NA	1.14 ±0.20	(0, 9.73)	0.04 ±0.005	(0, 0.23)
Cn	NA	NA	76.94 ±5.41	(0.27, 268.29)	200.69 ±7.07	(85.70, 404.50)
E2	NA	NA	57.47 ±1.84	(12.57, 113.46)	94.15 ±1.85	(55.12, 147.10)
Effegi	60.67 ±2.31	(22.24, 120.77)	NA	NA	NA	NA
M1	NA	NA	50.41 ±1.62	(9.36, 113.39)	70.09 ±1.45	(39.37, 124.38)
M2	NA	NA	59.05 ±1.99	(5.83, 114.90)	98.94 ±1.82	(62.23, 150.01)
OAH	NA	NA	41.53 ±1.32	(8.344, 83.56)	68.02 ±1.15	(41.85, 104.47)
OMH	NA	NA	61.38 ±1.86	(13.44, 119.94)	99.02 ±1.82	(62.23, 150.01)
QF	NA	NA	-52.16 ±7.59	(-335, 147.91)	67.29 ±5.09	(-78.56, 201.92)
Firmness	1.90 ±0.11	(1, 3)	2.28 ±0.07	(1, 5)	2.73 ±0.07	(1, 4)
Crispness	1.70 ±0.10	(1, 3)	2.27 ±0.08	(1, 5)	2.45 ±0.06	(1, 4)
Juiciness	1.74 ±0.10	(1, 3)	2.53 ±0.07	(1, 4.5)	2.25 ±0.06	(1, 4)

Table 2.2 continued (5 of 6)

	2010					
Trait[z]	**CU**		**UMN**		**WSU**	
20-week storage						
A1	NA	NA	32.84 ±2.45	(11.34, 67.63)	38.86 ±0.85	(21.52, 59.02)
A2	NA	NA	58.9 ±3.6	(29.14, 105.97)	72.49 ±1.45	(43.09, 109.22)
C0	NA	NA	0.19 ±0.08	(0, 1.95)	0.06 ±0.01	(0, 0.26)
Cn	NA	NA	108.35 ±13.49	(13.6, 250.50)	178.56 ±7.67	(54.01, 408.31)
E2	NA	NA	63.43 ±3.82	(29.67, 99.14)	83.94 ±1.99	(36.48, 136.34)
Effegi	56.88 ± 5.74	(26.91, 91.18)	NA	NA	NA	NA
M1	NA	NA	58.37 ±4.80	(19.55, 129.59)	60.83 ±1.43	(33.81, 97.16)
M2	NA	NA	69.3 ±4.53	(34.03, 133.28)	88.77 ±1.91	(49.97, 138.44)
OAH	NA	NA	32.65 ±7.82	(-65.3, 90.93)	61.03 ±1.25	(35.92, 91.19)
OMH	NA	NA	69.77 ±4.53	(34.03, 134.57)	88.77 ±1.91	(49.97, 138.44)
QF	NA	NA	-22.15 ±18.32	(-216.37, 143.78)	51.77 ±4.12	(0.16, 164.86)
Firmness	2.21 ±0.14	(1, 4)	2.72 ±0.21	(1, 4.5)	2.06 ±0.08	(1, 3.5)
Crispness	2.24 ±0.15	(1, 4)	2.55 ±0.22	(1, 5)	1.97 ±0.08	(1, 3.5)
Juiciness	2.00 ±0.13	(1, 3)	2.74 ±0.10	(1.5, 3.5)	1.98 ±0.06	(1, 3)

Table 2.2 continued (6 of 6)

	2011					
Trait[z]	**CU**		**UMN**		**WSU**	
20-week storage						
A1	NA	NA	24.68 ±0.78	(2.23, 53.40)	40.67 ±0.86	(19.37, 60.54)
A2	NA	NA	43.13 ±1.58	(2.761, 82.81)	73.45 ±1.37	(44.28, 109.12)
C0	NA	NA	1.21 ±0.22	(0, 9.73)	0.06 ±0.01	(0, 0.29)
Cn	NA	NA	56.83 ±4.41	(0.02, 230.75)	186.59 ±8.16	(53.51, 570.53)
E2	NA	NA	37.98 ±2.25	(0, 93.73)	84.54 ±1.95	(47.13, 136.81)
Effegi	53.50 ±7.07	(25.35, 104.75)	NA	NA	NA	NA
M1	NA	NA	44.02 ±1.50	(3.31, 100.19)	62.92 ±1.35	(29.96, 92.58)
M2	NA	NA	49.91 ±1.87	(3.604, 97.18)	89.98 ±1.86	(50.72, 138.86)
OAH	NA	NA	34.74 ±1.20	(2.45, 68.4)	62.43 ±1.18	(35.21, 91.35)
OMH	NA	NA	52.35 ±1.72	(3.746, 101.35)	90.00 ±1.86	(50.72, 138.86)
QF	NA	NA	-82.83 ±7.5	(-329.81, 110.34)	39.36 ±5.81	(-144.97, 152.67)
Firmness	1.71 ±0.10	(1, 3)	2.12 ±0.07	(1, 4.25)	2.55 ±0.08	(1, 4)
Crispness	1.52 ±0.09	(1, 3.5)	2.07 ±0.07	(1, 4.75)	2.37 ±0.07	(1, 4)
Juiciness	1.59 ±0.10	(1, 3.5)	2.38 ±0.07	(1, 4)	2.17 ±0.06	(1, 3.5)

[z] Mohr Digi-Test traits A1, A2, E2, M1, M2, OAH, and OMH reported in N; C0 reported in cm; and QF and Cn are derived traits without units where high values correspond to high quality and high crispness, respectively. Sensory traits firmness, crispness and juiciness are assessed on a 1 to 5 scale where 1 is low and 5 is high.

Table 2.3. Year-to-year repeatability (shown by Pearson's correlations) of sensory (firmness, crispness, juiciness) and instrumental texture traits measured at harvest and after 10 or 20 weeks refrigerated storage followed by 1 week at room temperature at the University of Minnesota (UMN), Washington State University (WSU), and Cornell University (CU) . Data aggregated for all genotypes at each location. See text and Figure 2.1 for description of instrumental measures using Mohr Digi-Test (A1, A2, C0, Cn, E2, M1, M2, OAH, OMH, QF) and Effegi penetrometers.

Trait[z]	Location	Harvest	10-week storage	20-week storage
A1	**UMN**	0.64 ****	0.67 ***	0.88 ****
	WSU	0.65 ****	0.72 ****	0.71 ****
A2	**UMN**	0.54 ****	0.63 ***	0.86 ****
	WSU	0.62 ****	0.66 ****	0.72 ****
C0	**UMN**	0.39 **	-0.16^{NS}	-0.11^{NS}
	WSU	0.42 ***	0.73 ****	0.68 ****
Cn	**UMN**	0.47 ***	0.33^{NS}	0.29^{NS}
	WSU	0.37 ***	0.61 ****	0.49 ****
E2	**UMN**	0.49 ****	0.54 **	0.85 ****
	WSU	0.68 ****	0.66 ****	0.73 ****
M1	**UMN**	0.62 ****	0.71 ****	0.93 ****
	WSU	0.54 ****	0.65 ****	0.67 ****
M2	**UMN**	0.49 ****	0.53 **	0.84 ****
	WSU	0.66 ****	0.66 ****	0.72 ****
QF	**UMN**	0.54 ****	0.64 ***	0.85 ****
	WSU	0.61 ****	0.74 ****	0.48 ***
Effegi	**CU**	0.70 ****	0.71 ****	0.73 ***

Table 2.3 continued (2 of 2)

Trait[z]	Location	Harvest	10-week storage	20-week storage
Crispness	**UMN**	0.48 ***	0.56 **	0.78 ****
	WSU	0.13[NS]	0.37 **	0.66 ****
	CU	0.14[NS]	0.65 ***	0.59 **
Firmness	**UMN**	0.43 ***	0.75 ****	0.81 ****
	WSU	0.37 ***	0.37 **	0.46 ***
	CU	0.30[NS]	0.54 **	0.44 *
Juiciness	**UMN**	0.40 **	0.40 *	0.29[NS]
	WSU	0.29 **	0.38 **	0.35 **
	CU	0.19[NS]	0.65 ***	0.70 ***

[NS],*,**,***,**** non-significant and significant at $p \leq 0.05$, 0.01, 0.001, or 0.0001 respectively.

[z] Mohr Digi-Test traits A1, A2, E2, M1, M2, OAH, and OMH reported in N; C0 reported in cm; and QF and Cn are derived traits without units, while high values correspond to high quality and high crispness, respectively. Sensory traits firmness, crispness and juiciness are assessed on a 1 to 5 scale, with 1 corresponding to low and 5 to high.

Table 2.4. Spearman's rank order correlations between sensory and instrumental texture traits measured at harvest and after 10 or 20 weeks refrigerated storage followed by 1 week at room temperature at the University of Minnesota (UMN), Washington State University (WSU), and Cornell University (CU), at each location.

		2010, Harvest			2010, 10-week storage			2010, 20-week storage		
Trait[z]		**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**
A1	**WSU**	0.18 ^{NS}	0.49****	-0.42****	0.51****	0.62****	0.28*	0.65****	0.71****	0.37***
	UMN	0.40***	0.73****	0.12^{NS}	0.83****	0.82****	0.56***	0.85****	0.83****	0.50*
A2	**WSU**	0.065^{NS}	0.43 ****	-0.52 ****	0.48 ****	0.61 ****	0.29 **	0.68 ****	0.72 ****	0.42 ***
	UMN	0.33 **	0.68 ****	0.27 *	0.77 ****	0.76 ****	0.48 **	0.87 ****	0.85 ****	0.58 **
C0	**WSU**	-0.053^{NS}	-0.34 ***	0.48 ****	-0.42 ****	-0.58 ****	-0.15	-0.56 ****	-0.64 ****	-0.30 **
	UMN	-0.42 ***	-0.79 ****	-0.13^{NS}	-0.72 ****	-0.76 ****	-0.49 **	-0.67 ***	-0.84 ****	-0.27^{NS}
Cn	**WSU**	0.38 ***	0.38 ***	0.094^{NS}	0.51 ****	0.58****	0.52 ****	0.47 ****	0.45 ****	0.42 ***
	UMN	0.44 ****	0.34 **	0.42 ***	0.32^{NS}	0.28^{NS}	0.016^{NS}	0.34^{NS}	0.42 *	0.33^{NS}
E2	**WSU**	-0.069^{NS}	0.25 *	-0.43 ****	0.44 ****	0.53 ****	0.27 *	0.63 ****	0.66 ****	0.40 ***
	UMN	0.28 *	0.47 ****	0.42 ***	0.61 ***	0.62 ***	0.34^{NS}	0.87 ****	0.85 ****	0.62 *
Effegi	**CU**	0.47 ***	0.70 ****	0.13^{NS}	0.55 ***	0.77 ****	0.47 **	0.50 **	0.75 ****	0.40 *

z Mohr Digi-Test traits A1, A2, E2, M1, M2, OAH, and OMH reported in N; C0 reported in cm; and QF and Cn are derived traits without units, while high values correspond to high quality and high crispness, respectively. Sensory traits firmness, crispness and juiciness are assessed on a 1 to 5 scale, with 1 corresponding to low and 5 to high.

Table 2.4 continued (2 of 4)

		2010, Harvest			2010, 10-week storage			2010, 20-week storage		
Trait[z]		**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**
M1	**WSU**	0.089^{NS}	0.44 ****	-0.55 ****	0.42 ****	0.61 ****	0.23 *	0.65 ****	0.72 ****	0.38 ***
	UMN	0.38 ***	0.73 ****	0.051^{NS}	0.80 ****	0.86 ****	0.46 **	0.86 ****	0.84 ****	0.47 *
M2	**WSU**	0.0029^{NS}	0.34 **	-0.49 ****	0.46 ****	0.55 ****	0.31 **	0.63 ****	0.66 ****	0.40 ***
	UMN	0.31 **	0.46 ****	0.45 ****	0.59 ***	0.61 ***	0.35 *	0.84 ****	0.82 ****	0.62 **
OAH	**WSU**	0.10^{NS}	0.46****	-0.48****	0.51****	0.62****	0.32**	0.69****	0.72****	0.44****
	UMN	0.36**	0.68****	0.28*	0.77****	0.77****	0.47**	0.64***	0.72****	0.50*
OMH	**WSU**	0.0029^{NS}	0.34**	-0.49****	0.46****	0.55****	0.31**	0.63****	0.66****	0.40***
	UMN	0.32**	0.46*****	0.45****	0.61***	0.64****	0.38*	0.82****	0.87****	0.58**
QF	**WSU**	0.14^{NS}	0.48 ****	-0.45 *****	0.52 ****	0.67 ****	0.33 **	0.43 ****	0.44 ****	0.24 *
	UMN	0.43 ***	0.73 ****	0.30 *	0.79 ****	0.80 ****	0.47 **	0.87 ****	0.88 ****	0.51 *
Crispness	**WSU**		0.41 ****	0.35 ***		0.70 ****	0.58 ****		0.90 ****	0.75 ****
	UMN		0.54 ****	0.53 ****		0.81 ****	0.72 ****		0.85 ****	0.58 **
	CU		0.61 ****	0.70 ****		0.75 ****	0.73 ****		0.74 ****	0.77 ****
Firmness	**WSU**			0.035^{NS}			0.55 ****			0.69 ****
	UMN			0.22^{NS}			0.64 ****			0.51 *
	CU			0.28 *			0.54 ***			0.61 ***

Table 2.4 continued (3 of 4)

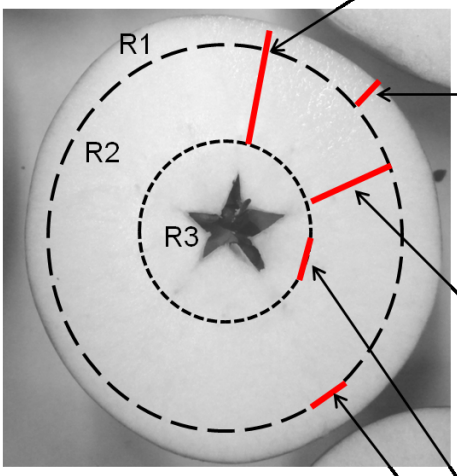
		2011, Harvest			2011, 10-week storage			2011, 20-week storage		
Trait[z]		**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**
A1	**WSU**	0.16^{NS}	0.38***	0.0041^{NS}	0.44****	0.61****	0.27**	0.61****	0.72****	0.42****
	UMN	0.36****	0.78****	0.091^{NS}	0.82****	0.84****	0.64****	0.79****	0.77****	0.60****
A2	**WSU**	0.12^{NS}	0.39 ****	-0.099^{NS}	0.47 ****	0.56 ****	0.20^{NS}	0.62 ****	0.74 ****	0.45 ****
	UMN	0.48 ****	0.78 ****	0.21 **	0.83 ****	0.85 ****	0.69 ****	0.75 ****	0.74 ****	0.67 ****
C0	**WSU**	0.010^{NS}	-0.31 **	0.10^{NS}	-0.46 ****	-0.62 ****	-0.20^{NS}	-0.59 ****	-0.67 ****	-0.38 ***
	UMN	-0.39 ****	-0.66 ****	-0.17 *	-0.68 ****	-0.70 ****	-0.59 ****	-0.66 ****	-0.68 ****	-0.60 ****
Cn	**WSU**	0.43 ****	0.20*	0.25*	0.35 ***	0.23 *	0.27 *	0.38 ***	0.37 ***	0.28 **
	UMN	0.47 ****	0.45 ****	0.31 ****	0.61 ****	0.61 ****	0.52 ****	0.54 ****	0.52 ****	0.44 ****
E2	**WSU**	0.12^{NS}	0.36 ***	-0.15^{NS}	0.44 ****	0.46 ****	0.23 *	0.53 ****	0.67 ****	0.43 ****
	UMN	0.50 ****	0.76 ****	0.23 **	0.83 ****	0.84 ****	0.68 ****	0.80 ****	0.78 ****	0.67 ****
Effegi	**CU**	0.19^{NS}	0.65 ****	0.091^{NS}	0.73 ****	0.86 ****	0.53 ****	0.58 ****	0.85 ****	0.61 ****

Table 2.4 continued (4 of 4)

		2011, Harvest			2011, 10-week storage			2011, 20-week storage		
Trait[z]		**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**	**Crispness**	**Firmness**	**Juiciness**
M1	**WSU**	0.078^{NS}	0.33 ***	-0.012^{NS}	0.44 ****	0.62 ****	0.22 *	0.63 ****	0.71 ****	0.43 ****
	UMN	0.29 ***	0.75 ****	0.034^{NS}	0.83 ****	0.88 ****	0.62 ****	0.82 ****	0.84 ****	0.57 ****
M2	**WSU**	0.13^{NS}	0.39 ****	-0.15^{NS}	0.46 ****	0.50 ****	0.20^{NS}	0.57 ****	0.68 ****	0.45 ****
	UMN	0.51 ****	0.77 ****	0.24 **	0.84 ****	0.85 ****	0.69 ****	0.75 ****	0.74 ****	0.65 ****
OAH	**WSU**	0.15^{NS}	0.39****	-0.075^{NS}	0.48****	0.58****	0.25*	0.65****	0.76****	0.47****
	UMN	0.47****	0.80****	0.23**	0.84****	0.86****	0.70****	0.82****	0.79****	0.68****
OMH	**WSU**	0.13^{NS}	0.39****	-0.14^{NS}	0.45****	0.51****	0.19^{NS}	0.57****	0.68****	0.45****
	UMN	0.50****	0.79****	0.24**	0.85****	0.87****	0.67****	0.82****	0.81****	0.65****
QF	**WSU**	0.19^{NS}	0.38 ***	-0.035^{NS}	0.50 ****	0.59 ****	0.24 *	0.63 ****	0.73 ****	0.44 ****
	UMN	0.49 ****	0.80 ****	0.23 **	0.83 ****	0.88 ****	0.63 ****	0.76 ****	0.77 ****	0.58 ****
Crispness	**WSU**		0.40 ****	0.49 ****		0.61 ****	0.49 ****		0.69 ****	0.72 ****
	UMN		0.45 ****	0.60 ****		0.84 ****	0.78 ****		0.88 ****	0.71 ****
	CU		0.33 *	0.68 ****		0.75 ****	0.79 ****		0.70 ****	0.82 ****
Firmness	**WSU**			0.20^{NS}			0.49 ****			0.57 ****
	UMN			0.13^{NS}			0.60 ****			0.58 ****
	CU			0.23^{NS}			0.54 ****			0.72 ****

^{NS},*,**,***,**** non-significant and significant at p≤ 0.05, 0.01, 0.001, or 0.0001 respectively.

Figure 2.1. Apple equatorial slice demonstrating MDT-1 fruit texture measures described by Mohr and Mohr (2000) and Evans et al. (2010). R1 is the outer area of the apple directly below the skin, R2 is the main edible portion of the fruit, and R3 contains the core. Bold lines indicate regions in which traits are determined.



Overall Maximum Hardness (OMH): largest recorded pressure

Overall Average Hardness (OAH): average pressure of entire test

Maximum Region 1 (M1): maximum pressure in first 8.1 mm

Average Region 1 (A1): average reading in first 8.1 mm

Maximum Region 2 (M2): maximum pressure between 8.1 mm and fruit core

Average Region 2 (A2): average reading from 8.1 mm to fruit core

Crispness Measurement (Cn): energy released upon fruit tearing, calculated from region 2 raw data

End Region 2 (E2): pressure at boundary of core

Creep (C0): deformation at boundary between regions 1 and 2, distance probe advances with constant force in set period of time

Quality Factor (QF): weighted index based on above traits

Figure 2.2. Proportions of variance attributable to year, individual, sampling and year×individual for fruit texture measures at three locations at harvest. ANOVA was used. Data from University of Minnesota (UMN), Washington State University (WSU), and Cornell University (CU) are shown. Abbreviations are as follows: A1, A2, average pressure regions 1 and 2, respectively (N); C0, creep at boundary between regions 1 and 2 (cm); Cn, crispness measurement (derived value); E2 pressure at core boundary (N); M1, M2, maximum pressure regions 1 and 2, respectively (N); OAH, overall average hardness (N); OMH, overall maximum hardness (N); and QF, quality factor (derived value). The sensory measures of crispness, firmness, and juiciness were assessed on a 5-point scale.

Figure 2.2 continued (2 of 2)

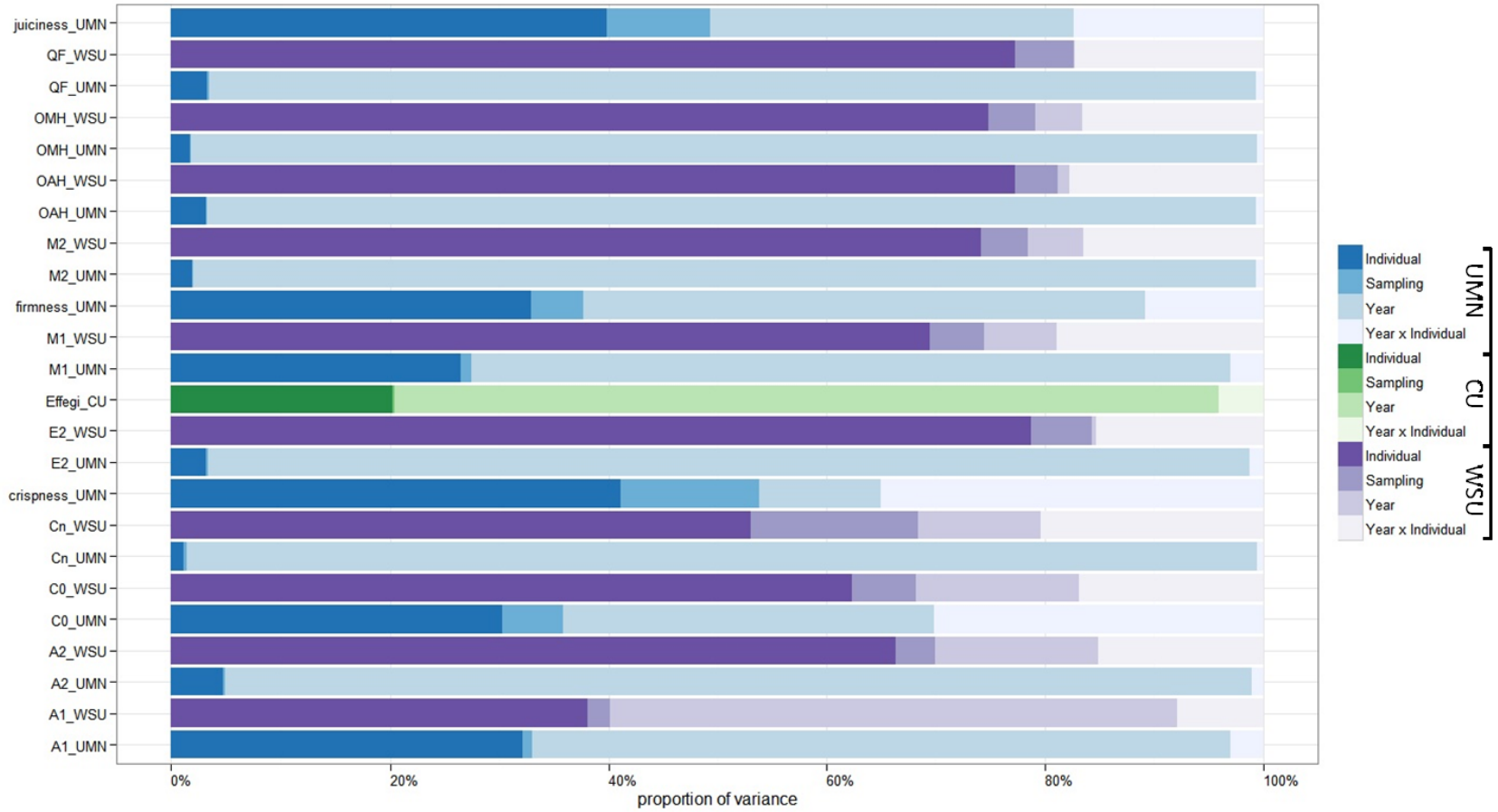


Figure 2.3. Bean plots of changes in sensory texture components from harvest to 10 weeks of storage (distributions to left of vertical lines) and from harvest to 20 weeks storage (distributions to the right of vertical lines) at University of Minnesota (UMN), Washington State University (WSU), and Cornell University (CU). Dashed horizontal lines mark year means across locations. Bold black horizontal lines mark the mean of each storage interval distribution. Minor black and white horizontal lines are individual observations, in which line width indicates multiple observations of the same value, in the format of a histogram; while filled areas are a density trace of the distribution. Sensory measures were assessed on a 5-point scale.

Figure 2.3 continued (2 of 2)

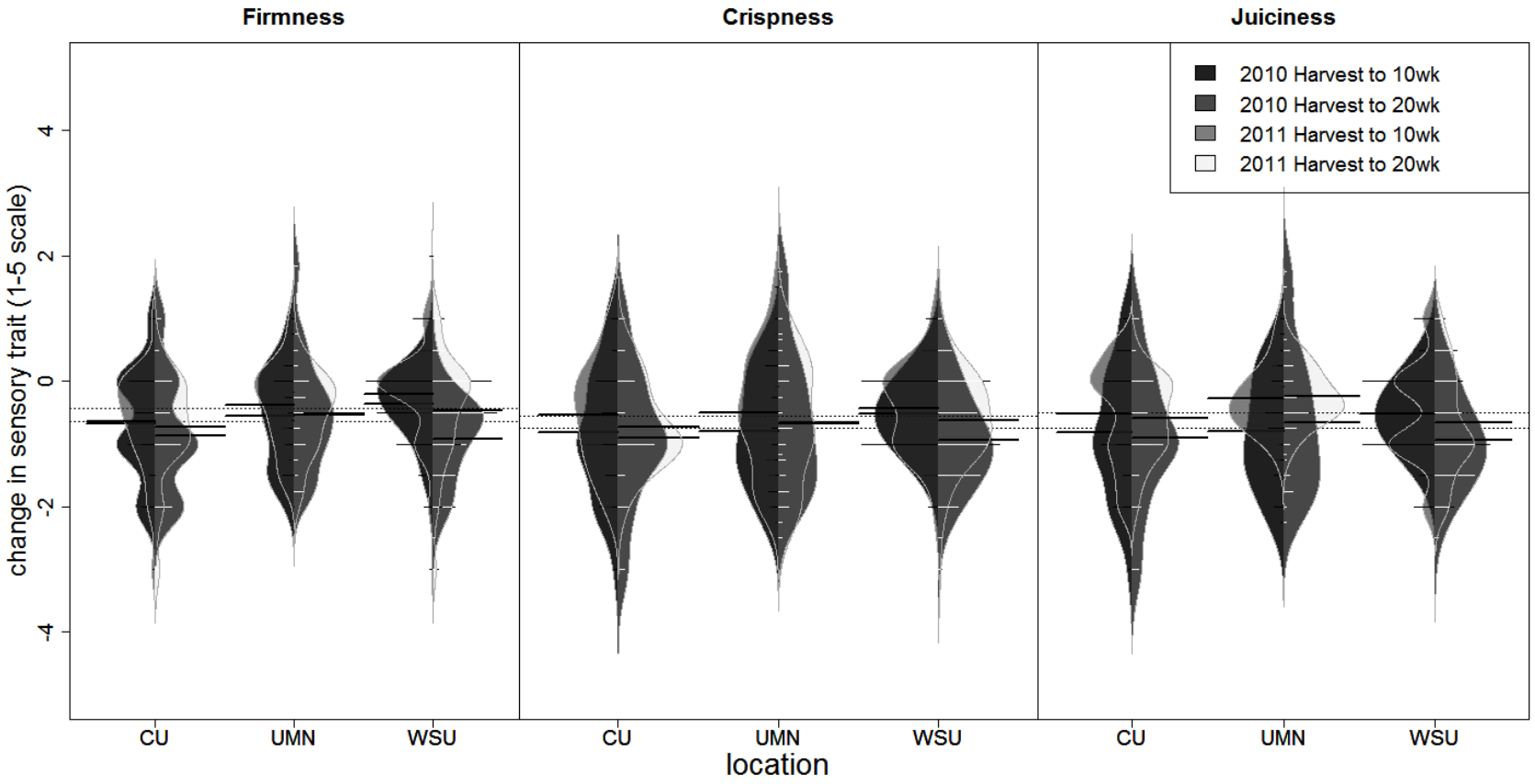
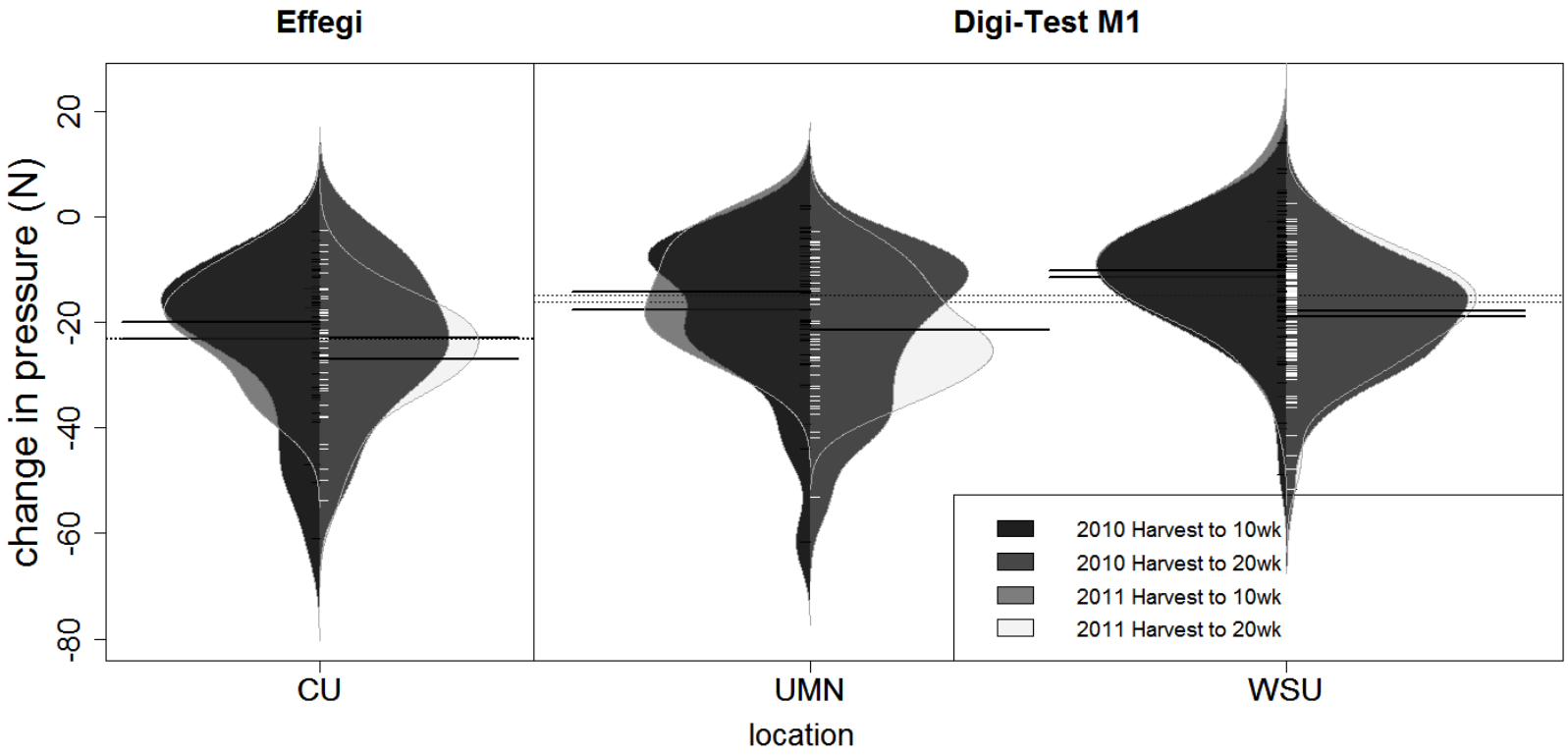


Figure 2.4. Observed changes in instrumental firmness over storage at University of Minnesota (UMN), Washington State University (WSU), and Cornell University (CU). Dashed horizontal lines mark year averages across locations. Bold black horizontal lines mark the mean of each distribution Bold black horizontal lines mark the mean of each storage interval distribution. Minor black and white horizontal lines are individual observations, in which line width indicates multiple observations of the same value, in the format of a histogram; while filled areas are a density trace of the distribution. M1 measured by Mohr Digi-Test-1 (Fig. 2.1) is reportedly equivalent to firmness measured with an Effegi penetrometer (Mohr and Mohr, 2000).

Figure 2.4 continued (2 of 2)



CHAPTER THREE: A CONSENSUS 'HONEYCRISP' APPLE (*Malus* × *domestica*) GENETIC LINKAGE MAP FROM THREE FULL-SIB PROGENY POPULATIONS

Introduction

'Honeycrisp' is an emerging apple cultivar with increased importance in North America due to its outstanding flavor and textural traits (Hoover et al. 2000; Luby and Bedford 1992; Tong et al. 1999). Although it is prone to some storage disorders, 'Honeycrisp' can maintain crispness for 6-9 months in storage (Luby and Bedford 1992; Tong et al. 1999). Furthermore, 'Honeycrisp' has been shown to exhibit field resistance to foliar apple scab infection when grown under organic disease management practices (Berkett 2007). This characteristic is important for 'Honeycrisp' growers who may be able to reduce fungicide inputs in their orchards. For the apple breeder, using 'Honeycrisp' as a parent offers the genetic background for superb fruit quality and disease resistance traits that should be leveraged in breeding (McKay et al. 2011). Identifying the marker-locus-trait associations in 'Honeycrisp' progeny will give plant breeders additional tools for marker assisted selection (MAS), and marker assisted breeding (MAB), in developing new cultivars. The development of a 'Honeycrisp' linkage map will add to the toolbox available to apple breeders and geneticists.

Fruit quality traits are among the most important characteristics evaluated and the most crucial component of a breeding project as the fruit are the saleable product driven by consumer demand. These quality traits include texture (King et al. 2000) and its

components firmness (Pre-Aymard et al., 2005), juiciness, and crispness. The development of scab resistant cultivars faces genetic challenges (linkage drag) and marketing challenges. Any new cultivar must be an outstanding alternative or replacement to an existing, consumer-recognized cultivar. Consumer familiarity with a cultivar and previous purchase of a particular apple cultivar rank as top determinants in selecting fruit to purchase (Kelley et al., 2010).

Several constraints confront the apple breeder in producing new cultivars that meet consumer and grower needs. Cultivar development is hindered by long juvenility and self-incompatibility which constrain crossing decisions. The development of a single cultivar can take as many as 20-25 years. Due to the large size of mature trees, orchard space is limited, and the maintenance of individual trees from juvenility to fruit-bearing age is expensive and requires a large amount of space. The development of genetic markers to screen important traits at the seedling stage and for parental selection will result in the enrichment of the target trait among seedlings that are grown to maturity for phenotypic evaluation. Accurate phenotyping of the traits of interest predicates detection of robust marker-trait associations to enable MAB (Luby and Shaw, 2001). The traits must be well defined and also objectively measurable.

Apples are asexually propagated and are grown on rootstocks to allow multiple growers to have an infinite number of trees of the same genotype. However, in an apple breeding program, it is often not practical or economical to have multiple replications of the same genotype, especially if the individual has not been proven desirable in preliminary taste and sensory evaluations. As a result, seedling evaluation occurs on a

single tree, grown in one location. In a woody species like apple with long generation times, obligate outcrossing nature, and long juvenility (Maliepaard et al., 1998) the development of mapping populations is time consuming and resource limiting if the progeny have little chance of becoming selected for cultivar development. The development of large mapping populations in apple is generally applied only to traits that can be screened at the seedling stage (e.g., disease resistance) and even then only select plants (e.g., disease resistant) are maintained into adulthood. As a result, mapping populations for genetic studies are created *ad hoc* from existing full-sib families that were developed over a number of years, often through reciprocal crosses, and planted throughout the orchard as space allowed.

Due to its highly heterozygous genome, high levels of inbreeding depression, and self-incompatibility, genetic studies in apple can be challenging (Lawson et al., 1995). Linkage maps for self-incompatible species, including apple, are created using the two way pseudo test-cross method within a single progeny (Grattapaglia and Sederoff, 1994). In this approach, a map for the first parent is made using makers heterozygous in the first parent and homozygous in the second, and conversely the second parental map consists of markers homozygous in the first parent and heterozygous in the second. These maps can then be integrated using markers heterozygous in both parents, creating a population map. Genetic mapping using bi-parental mapping populations is common in apple genetics, especially in developing molecular markers for monogenic traits such as disease resistance (Schenato et al., 2008; Tartarini and Sansavini, 2003). A number of linkage maps have been developed and were used to detect quantitative trait loci (QTLs) and map

genes for a range of important traits including disease resistance (*Vf* for apple scab (Gianfranceschi et al., 1996)), acidity (*Ma*; Maliepaard et al., 1998), and growth habit and developmental traits (Lawson et al., 1995). Mapping populations typically use parents divergent for an important trait. This includes recently published microsatellite and single nucleotide polymorphism (SNP) maps in *Malus* species (Antanaviciute et al., 2012; Fernández-Fernández et al., 2012; Wang et al., 2011). The advantage of a consensus 'Honeycrisp' linkage map is that marker alleles in this cultivar would yield a novel map that would be informative for MAB in breeding programs using this cultivar.

Recently, the apple genome was sequenced (Velasco et al., 2010), and additional supporting tools have been developed, including a physical map, BLAST search engine, and genome browser available on Genome Database for Rosaceae (GDR; http://www.rosaceae.org, Jung et al., 2008). High throughput SNP genotyping allows for efficient genotyping of large numbers of individuals or populations with a relatively low cost per marker. An 8K SNP array v1 was developed by the International RosBREED SNP Consortium (IRSC). Based on the Illumina Infinium platform, the BeadChip is a small, portable, highly repeatable assay that allows for rapid scoring of individuals, providing even coverage throughout the apple genome, including SNPs within putative expressed genes (Chagné et al., 2012). The array was designed using a clustering strategy with a cluster of 4-10 closely positioned SNPs spaced at 1cM intervals between clusters (Chagné et al., 2012). Clustered markers should provide local information for diverse apple populations representing unique haplotypes, and recombination is rarely expected within a cluster. The result is a SNP array that is not population dependent and

is applicable across cultivars and progeny populations (Micheletti et al., 2011).

Linkage maps with dense marker coverage and with markers evenly spaced across the genome are ideal for QTL analysis. Increased density and coverage of markers helps increase power and precision of QTL analysis, thereby helping in gene discovery. Khan et al. (2012) created a highly saturated map of apple by merging five bi-parental maps by use of single sequence repeat (SSR) and SNP markers shared among the linkage maps. The construction and analysis of genetic linkage maps provide support for the placement of molecular markers into the correct order and position. The correct order and position is very important to precisely locate QTLs. A constraint in map construction is marker checking to validate and correct automated SNP genotyping calls, especially in cases of expected paralogous regions from local or whole genome duplication events, which are common in plant genomes, including those of *Malus* species (Velasco et al., 2010). A comparative analysis of maps from different populations and development of a consensus map help to determine whether large genome rearrangements are present and to establish consensus order and positions of mapped markers.

The objective of this study was to develop a high-density, SNP consensus linkage map for 'Honeycrisp' utilizing several 'Honeycrisp' full-sib progeny populations that segregate for fruit quality and apple scab resistance. This map will provide the framework for future genetic studies in 'Honeycrisp'-specific progeny to identify marker-locus-trait associations for important fruit quality and disease resistance traits, thus enabling MAS and MAB. It will also provide additional support in map construction (marker order and position) for pedigree-based analysis and in resolving potential issues

in the apple physical map v1 (GDR database: http://www.rosaceae.org; Jung et al., 2008).

Materials and Methods

Plant Materials. A portion of the genotypic data in this study was produced as part of the RosBREED crop reference set (rosbreed.org). The corresponding apple genotypes, hereafter referred to as "RosBREED samples", included the parents ('Honeycrisp', 'Gala Twin Bee', 'Monark', MN1764, and 21 individuals of the 'Honeycrisp' × 'Monark' population (described below). The majority of individuals described in this paper were genotyped independently of the RosBREED crop reference set and these individuals are hereafter referred to as "UMN samples".

The UMN samples comprise three full-sib families sharing 'Honeycrisp' as a common parent and were utilized in the development of the 'Honeycrisp' consensus map. Two *ad hoc* populations ('Honeycrisp' × MN1764 (n=112) and 'Honeycrisp' × 'Monark' (n=81), individuals resulted from crosses made both directions in each population) were selected from breeding populations growing at the University of Minnesota Horticultural Research Center (Excelsior and Chanhassen, MN) that were developed from crosses made in 1992-1998. These *ad hoc* populations have been described previously by McKay et al. (2011). A third population was created in 2010 from a cross of 'Honeycrisp' × 'Gala Twin Bee' (n=125; this populations is referred to as 'Honeycrisp' ×

'Gala' throughout) and grown in greenhouses at the University of Minnesota-Twin Cities (St. Paul, MN).

DNA Extraction Protocol. For RosBREED samples, stems with newly expanding leaf tissue were collected in the field in 2010 and 2011 and placed in labeled plastic bags on ice. Thirty to 50 mg of leaf tissue were later harvested into a cluster tube (Corning, Tewsbary, MA). These RosBREED tissue samples were frozen in liquid nitrogen and held at -80°C until DNA extraction.

For the UMN samples, newly expanding leaves were collected (when available) from individual trees for DNA extraction in 2012. If unfurling leaves were not available, the youngest and/or smallest leaves were collected. Leaf tissue was placed into labeled paper coin envelopes, held on dry ice during collection, and then frozen at -80° C for at least 12 hours. Frozen tissue was lyophilized in envelopes in small batches for at least two days until a constant mass was reached for an individual sample. Lyophilized tissues in coin envelopes were stored in plastic zip-top bags with Drierite (W.A. Hammond Drierite Co. Ltd, Xenia OH) at -80° C. Approximately 10 to 15 mg of lyophilized leaf tissue from each sample was placed into a cluster tube. The racks of tubes containing samples were set in a zip-top bag with Drierite overnight before sealing with strip caps. Samples were stored in 96-cluster racks in zip-top bags with Drierite at -80 °C until DNA extraction.

Leaf tissue was homogenized by grinding lyophilized (UMN) or frozen (RosBREED) samples. A 4 mm stainless steel bead (McGuire Bearing Company, Salem,

OR) was added to each cluster tube. New caps were applied and the 96-tube rack was submerged in liquid nitrogen. The rack was then placed into a Retsch MM301 Mixer Mill (Retch, Haan, Germany) and shaken for 30 seconds. Sample racks were re-submerged in liquid nitrogen and shaken two additional times, disrupting the leaf tissue into a fine powder. Strip caps were secured frequently to prevent contamination. The homogenized RosBREED and UMN tissue was stored at -80° C until 10 minutes prior to extraction.

Extraction was conducted using the E-Z 96® Plant DNA Kit (Omega Biotek, Norcross, GA) with modifications (Gilmore et al. 2011). Additional modifications from these protocols included using SP1 solution equilibrated to 65° C in a water bath. The supernatant (580 µL) for each sample was transferred in one step to a new cluster tube containing 10 µL RNase solution (2.5 µL RNase and 7.4 µL Tris EDTA (TE) buffer, pH 8.0). After the drying step, DNA was eluted in 100 µL elution buffer, and samples were stored at 4° C and quantified within seven days, or stored at -20° C.

DNA Quantitation using PicoGreen Assay. DNA samples were quantitified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Eugene, OR) and a Victor multi-plate reader (Perkin Elmer Inc., San Jose, CA, USA). Target concentrations were generally between 50-100 ng/µL. Samples with DNA concentration below 20 ng/µL were re-extracted. Samples with DNA concentrations > 100 ng/µL were diluted with the addition of an equal volume of TE to achieve concentrations between 50-100 ng/µL. Fifteen µL of

each DNA sample were aliquoted into 0.2 mL PCR plates. Plates were sealed with adhesive aluminum foil seals for shipment on dry ice to the genotyping facility.

*Marker Data Generation and Analysis.*The UMN DNA samples were submitted to the SNP Genotyping Facility at Michigan State University (East Lansing, MI). The RosBREED samples were analyzed at the University of Western Cape, South Africa. Using previously published protocols (Illumina, 2006a) samples were hybridized onto the International RosBREED SNP Consortium (IRSC) apple 8K SNP array v1 (Chagné et al., 2012) following a whole genome amplification reaction. BeadChips were imaged by the iSCAN system and converted into intensity data. The intensity data from the two data sets were combined for analysis and interpretation in the Genotyping Module of GenomeStudio for genotype clustering (Illumina Inc., 2010a).

The iSCAN data from both genotyping facilities were loaded into a single project file for data analysis. SNP genotype scoring employed the Genotyping module of GenomeStudio (Illumina Inc., 2010b) software version v2010.3.0.30128. The software normalizes the intensity values across BeadChips to allow for uniformity in allele-calling. To ensure high quality reads, stringent initial parameters were set as: GenTrain >0.60 and AB Freq from 0.45 to 0.55. The SNPs were clustered by marker locus using the clustering algorithm Gentrain2 (Illumina Inc., 2010c) and all SNPs were visually examined for an expected maximum of three clusters (AA, AB, and BB) and then classified as failed, monomorphic, or polymorphic.

Automated allele calling with visual checking to confirm clustering of individuals

into appropriate classes was utilized. Manual clustering was performed for some markers, when automated clustering was not satisfactory. Markers with more than three distinctly-spaced clusters, presumably the result of annealing to more than one genomic region (i.e. paralogs), were excluded. The 'Honeycrisp' × MN1764 population, was utilized to select nearly 2000 high quality markers for the development of a saturated linkage map as suggested by Micheletti et al. (2011). A preliminary map was developed to evaluate genome coverage and relative positions in comparison to the physical map (Clark et al., 2013). For the preliminary map, the default settings of the maximum likelihood method in JoinMap 4.1 (Kyazama B.V. Wageningen, Netherlands; Van Ooijen 2006) were used to map 1952 SNP markers. Marker grouping during map construction utilized a published SNP map (Antanaviciute et al., 2012). These ~2000 markers were then scored for the 'Honeycrisp' × 'Gala' and 'Honeycrisp' × 'Monark' populations. Data were converted into biallelic codes (AA, AB or BB) at each marker locus.

Marker loci at which missing parental genotypes could not be positively determined based on progeny segregation in two or more families were removed. Markers with >10% missing data were eliminated. Progeny that did not conform to the parental genotypes were removed, as they were expected to be outcrosses, non-progeny, or contaminated samples. Progeny genotypic scores identified as genotyping error were considered as missing. The identity by descent (IBD) analysis program within FlexQTL™ was used to identify miscalled alleles and impute parental genotypes using the 'Golden Delicious' physical map positions. This tool allowed for the aggressive detection of errors (missing markers, null-alleles, other anomalies), but required

additional manual correction or imputation of parental genotypic scores based on the progeny SNP calls.

Linkage Mapping. The codominant SNP markers from each outbreeding, full-sib population were coded for linkage map construction according to JoinMap 4.1 conventions as heterozygous in either first or second parent (<nn x np>, <lm x ll>) or both parents (<hk x hk>) (Van Ooijen 2011). Initially, the three populations were mapped separately. The initial grouping procedure in JoinMap was completed using the published M432 progeny linkage map (Antanaviciute et al. 2012), resulting in a large proportion of the called SNPs remaining ungrouped and subsequently unmapped. The strongest crosslink values (SCL) were applied repeatedly using restrictively lower values in an iterative process to assign ungrouped loci to the correct linkage group (LG). Markers with suspect linkage (recombination frequency estimate >0.6) were removed before mapping. Then, map order was calculated using the maximum likelihood option for calculating marker order of both parental maps and an integrated map. For this study, only the single parent 'Honeycrisp' map from each population was used for the construction of the consensus map.

Each 'Honeycrisp' map and corresponding progeny genotypic data set were assembled for analysis in FlexQTL™ (Bink et al. 2008) to determine differences between observed double recombinant (oDR) frequency and expected double recombinants (eDR) frequency provided the newly constructed linkage map. We calculated oDR frequency minus eDR frequency for the two parents at each marker position. This helped to identify

markers that had high genotyping error rates or that were misplaced by the mapping algorithm. Markers with oDR-eDR ≥ 0.03 were removed from the subsequent round of JoinMap mapping, eliminating 100 (‘Honeycrisp’ × MN1764), 80 (‘Honeycrisp’ × ‘Gala’), and 105 (‘Honeycrisp’ × ‘Monark’) spuriously placed markers. The mapping steps listed above were repeated. After two rounds of mapping and removal of suspect markers identified with FlexQTL™, maps were inspected for large gaps (> 15 cM). Markers creating unusually large gaps at linkage group ends were referred to as “lone wolf” markers as the gaps suggested poor linkage to the marker group. If a large gap existed at the end of a LG in a single population map and the causative marker was not found in the corresponding LG in either of the other two maps, it was removed. After marker removal from any map, the map was recalculated in JoinMap 4.1. The resulting three ‘Honeycrisp’ maps were combined into a consensus map with the MergeMap (2012 version) software tool (Wu et al. 2011). Maps were weighted based on population size (‘Honeycrisp’ × ‘Monark’: 0.255, ‘Honeycrisp’ × ‘Gala’: 0.393, ‘Honeycrisp’ × MN1764: 0.352).

The consensus ‘Honeycrisp’ linkage map was compared to the available physical map of apple. SNP map positions for each of the 17 linkage groups were plotted against marker positions in the respective pseudo-chromosomes of the ‘Golden Delicious’ genome sequence with R v2.15.1 (R Core Team, 2012). Base pair positions were those of the mapped International Rosaceae SNP (IRSC) Apple SNP Infinium Array v1 markers and these data are available at the Genome Database for Rosaceae (http://www.rosaceae.org; accessed 28 Feb 2013). Each marker included in the consensus

'Honeycrisp' map was checked for significant segregation distortion (χ^2, $p < 0.005$) in each of the three families using JoinMap.

Results

Work flow and total quality SNP markers remaining at each phase are shown in Figure 3.1. The heterozygosity observed in each of the parents crossed with 'Honeycrisp' allowed for the construction of three 'Honeycrisp' linkage maps. Figure 3.2 details heterozygosity for each parent ('Honeycrisp' is heterozygous at each marker position) along the consensus map. Lack of heterozygosity along a linkage group for all three populations resulted in large gaps as indicated on the linkage map. MN1764 had the lowest proportion of heterozygous markers (34.5%) in the corresponding 'Honeycrisp' parental map, and MN1764 additionally had the lowest proportion of heterozygous markers in the consensus map (32.9%) (Table 3.1). The highest proportion of heterozygous markers was in the 'Honeycrisp' × 'Monark' population with 48.4% in the parental map and 45.2% in the consensus map. The 'Honeycrisp' × 'Gala' population had 33.0% heterozygous markers in the parental map and 31.0% in the consensus map.

Parental Linkage Maps. Three 'Honeycrisp' (single parent) linkage maps were constructed from segregating populations using SNP markers (Figure 3.3; Table_S1.xlsx). The maps each contained 17 linkage groups representing the 17 known chromosomes that comprise the *Malus* × *domestica* genome. The shortest map was 1097.55 cM and was constructed from the 'Honeycrisp' × 'Gala' population from 1042

markers with an average spacing of 1.05 cM between markers. The next longest map was 1340.20 cM and was constructed from the 'Honeycrisp' × 'Monark' population with 1018 SNP markers and an average marker spacing of 1.32 cM between markers (Table 3.2). The 'Honeycrisp' × MN1764 map was 1350.29 cM in length and was constructed from 1041 SNP markers, with an average marker spacing of 1.30 cM. The marker coverage for the linkage groups ranged from 23 markers (LG7 ('Honeycrisp' × MN1764)) to 88 markers (LG4 ('Honeycrisp' × MN1764 and 'Honeycrisp' × 'Monark')). The maximum gap size for any linkage group ranged from 5.13 cM (LG9 'Honeycrisp' × 'Gala') to 129.64 cM (LG17 'Honeycrisp' × 'Monark'). The "lone wolf" marker on LG17 of the 'Monark' map (refer to Figure 3.3) was retained as it met the parameters described above and was resolved in the consensus map.

Consensus Linkage Map. The three 'Honeycrisp' linkage maps were merged to create one consensus linkage map comprising markers segregating in one or more of the 'Honeycrisp' mapping populations (Figure 3.4). The consensus map was constructed using 1091 SNP markers (13.9% of the IRSC 8K SNP array v1; Table 3.2; Table_S2.xlsx). Figure 3.5 details the 951 markers in common across all three populations, and the 140 SNP markers segregating in only one or two populations. The consensus map is 1481.72 cM with an average distance of 1.36 cM between markers (Table 3.2). The sizes of the linkage groups range from 61.58 cM (LG8) to 130.48 cM (LG15). The largest gap in the linkage map was 34.21 cM on LG7.

Comparison of Genetic Positions to Physical Map. The genetic positions of markers in the consensus 'Honeycrisp' map were plotted against the physical positions of marker loci on the 'Golden Delicious' genome (Figure 3.6). Generally, there was agreement in the placement of the markers between the 'Honeycrisp' map and the genome sequence as evidenced by the linearity in the plots. The majority of the markers revealed direct correspondence between the linkage groups and the 'Golden Delicious' pseudo-chromosomes. Across the linkage map, 111 (10.2%) markers mapped to linkage groups other than the corresponding pseudo-chromosome. Seven markers that were placed in the consensus 'Honeycrisp' map were classified as "unanchored" in the physical map. Areas of high recombination, indicated by large horizontal gaps in Figure 3.6, were detected along several of the LGs including LGs 1, 6, 7, and 10. Areas of low recombination are also evident as marker clusters.

Segregation Distortion. Of the markers included in the consensus linkage map, 57 showed significant ($p < 0.005$) segregation distortion in the 'Honeycrisp' × 'Gala' progeny, 58 were significantly distorted in the 'Honeycrisp' × MN1764 progeny, and 41 were significantly distorted in 'Honeycrisp' × 'Monark' progeny. In total, only nine markers showed significant segregation distortion in two families (black points, Figure 3.6) and 138 markers showed significant segregation distortion in only one family (gray points, Figure 3.6). None of the markers of the consensus map showed significant segregation distortion at the 0.005 level in all three progenies. Of mapped markers,

13.5% showed significant segregation distortion. Significant distortion was primarily clustered to regions on LG2, LG5, LG6, LG13, LG14 and LG17.

Discussion

We have developed a consensus 'Honeycrisp' linkage map spanning 17 linkage groups representing the 17 chromosomes in the apple genome using the high-throughput IRSC 8k SNP array v1 (Chagné et al. 2012) and three mapping populations. The strategy utilized stringent data checking steps to ensure quality marker data including: selection of high quality SNP reads, removal of markers demonstrating a high frequency of double recombination, and examination of "lone wolf" markers. By analyzing each family separately, FlexQTL™ adequately identified problematic markers that did not meet expected and observed double recombinant frequency. We were not able to position these markers elsewhere in the map using Joinmap. The genomeim.csv file, from FlexQTL™, was easily manipulated to calculate and identify spurious markers that caused observation of double recombinants, negatively impacting linkage map construction. The double recombination pattern was visualized in Map Chart v2.2 (Voorrips 2002) and also provided a quick, graphical interpretation after each round of mapping. This method was convenient and intuitive without the added complexity of graphical genotyping for ordering markers and identifying spurious markers. This methodology utilizes files that can be used in QTL analysis with FlexQTL™, thus reducing the burden of creating new files or data for other interfaces.

The mapping approach outlined here drastically reduced the number of SNP markers to only 13.8% of those on the IRSC SNP array and 19.6% of total polymorphic markers on the array. The first reduction, to ~2000 (25%) SNP markers, was based on stringent parameters to identify high quality reads with visually distinguishable clusters in the GenomeStudio software. These markers were then scored for all three populations in accordance with other reports using similar numbers of markers for linkage mapping in apple (Antanaviciute et al. 2012; Micheletti et al. 2011). The FlexQTL™ inheritance checking algorithm efficiently identified problematic markers or inheritance errors. Data free of genotyping errors are very important for construction of genetic maps to ensure proper marker ordering.

The overall reduction in the number of markers is the result of stringent parameters utilized throughout the mapping strategy to ensure high quality data in the construction of a consensus linkage map with informative meiosis in 'Honeycrisp'. The detection of functional 'Honeycrisp' haplotypes will provide utility in genetic studies of progeny populations with the aim of identifying genetic contributions specific to this parent. The reduced number of markers in the map will be less computationally demanding for downstream software applications in the detection of QTL, as opposed to using all informative markers of the array and using the physical map in lieu of a genetic map. The consensus map has an average interval of 1.36 cM between markers, a much higher marker density than has been achieved for conventional SSR or other marker-based linkage maps, and provides sufficient marker coverage for moderate sized QTL mapping populations. The often touted advantage of a high-throughput SNP array is the

reduced price per marker. But marker quality and usefulness are not uniform across all loci. Homozygosity at a marker locus, genotyping quality, and genotyping errors all contribute to increasing the cost per informative marker. The development of a reduced array that retains polymorphic markers across the genome could reduce some of the cost and time spent resulting from lower quality SNP markers. Chagné et al (2012) showed that of the 8K array, only 70.6% of the markers were polymorphic in the > 1600 individuals, accessions and segregation populations that were evaluated. In an era where low genotyping costs efficiently enable an abundance of data, it is imperative to consider which data points are useful and develop affordable arrays that capture meaningful bioinformatic data.

Linkage mapping in JoinMap 4.1 utilizing the published M432 map (Antanaviciute et al., 2012) for the grouping step was computationally efficient. The multipoint maximum likelihood method for mapping was faster than regression mapping (Van Ooijen 2011) and was thus utilized in this study of outcrossing populations. The construction of two parental maps and an integrated map for each population was useful in determining the fate of "lone wolf" markers although only the 'Honeycrisp' parental map was retained for consensus map construction.

The three 'Honeycrisp' parental maps were unique and allowed the incorporation of unique markers due to observed differences in heterozygosity in the parents. For example, the distal end of LG15 also clearly shows how the consensus map was greatly extended by the inclusion of the 'Gala' and 'Monark' populations with the MN1764 population, which was homozygous for those markers (Figure 2). However, low levels of

heterozygosity were observed in some areas such as LG7, similar to the M432 map (Antanaviciute et al., 2012). To increase coverage in these regions, one could return to GenomeStudio and use less stringent quality parameters for SNP calls. Additionally, markers developed specifically from pseudo-chromosome 7 could be scored and added to the maps. Genomic regions with high levels of homozygosity shared among cultivars could be an artifact of domestication, genetic drift, other selection, or a bottleneck. An exploration of these areas among other cultivars and *Malus* species linkage maps could provide insight into the genes that reside in these areas.

The clustering strategy that was utilized in the development of the IRSC 8k SNP array resulted in many SNP markers mapping to the same locus. Low recombination in these areas makes it difficult to assign the correct map order. Observed differences in local homology between the parental maps may be the result of within cluster ordering. Using the physical map to order the markers would be one strategy to resolve this issue, however the ordering of the physical map may also be incorrect. Additionally, the physical order of markers may be different between the three populations due to disruption in micro-synteny and structural variations (Khan et al., 2012). Because the recombination frequency is so small within a cluster or tightly mapped clusters/markers, the precise order may not serve as a barrier to QTL detection. This is especially true in a pedigree-based approach, in which markers within a cluster may have different utility for individuals of different subpopulations. That is, any given individual SNP marker within a cluster at a single marker locus may segregate for some individuals or subpopulations and not others, but the map position is not lost for the entire pedigreed population.

Additionally, local marker order may not be important in establishing functional haplotypes in a cluster in which low frequencies of recombination events occur in the region.

Antanaviciute et al. (2012) compared map positions of an integrated apple rootstock linkage map to the 'Golden Delicious' genome sequence, reporting that 13.7% of genetically mapped markers did not associate with the predicted pseudo-chromosome. Our results are consistent with this finding, but may be influenced by our use of the M432 map for grouping of markers. For instance, a cluster of markers initially associated with pseudo-chromosome 9 of 'Golden Delicious' maps to the top of LG4 in both the M432 and 'Honeycrisp' maps. However, had our data not supported these placements, it is likely the markers would have been identified as "suspect linkages" during mapping and thus been discarded.

Significant segregation distortion was observed for 13.5% of the markers in the final 'Honeycrisp' consensus map when no quality control measures regarding segregation distortion were used during marker checking or linkage map construction. The choice not to use segregation distortion as a quality control measure was made because marker segregation distortion could represent real, biologically relevant segregation distortion. Largely supporting this hypothesis is the observation that markers exhibiting segregation distortion mapped in cohesive clusters along only a couple of linkage groups. Biological reasons for segregation distortion are those that impose selection upon the population such as selective fertilization (apple's gametophytic self-incompatibility), abortion of gametes (Liebhard et al., 2003), and other unavoidable

natural selective pressures such as field environment (e.g. winter hardiness) that are inadvertently imposed upon the breeding populations (i.e. the *ad hoc* mapping populations utilized in this study). Markers with observed segregation distortion need not be within the survival gene, and they may be linked with the gene conferring survivorship. Segregation distortion observed in this study was not found in the same linkage groups as that reported by Antanaviciute et al. (2012) with the exception of that on LG17 which contains the *S*-locus (Maliepaard et al. 1998).

The GenomeStudio software and manual calling of SNPs into biallelic clusters (AA, AB or BB) is constrained by the quality of reads. Inherent in difficulty with read quality are errors resulting from DNA quality, contamination, DNA hybridization and extension, and fluorescence signal. Recent whole genome duplication, segmental duplication, and a high degree of homology between some markers results in SNP markers exhibiting segregation behavior similar to that of polyploids in the cluster plots (Voorrips et al. 2011; personal observation). DNA from different genomic regions may hybridize to the same marker, typically resulting in more than three clusters. However, not all of these occurrences may be detected manually or within the automated calling. The spread of a cluster in automated/manual calling of multiple populations (pedigrees, diverse sets) may provide statistical support of a single cluster, but may mask the presence of more than three clusters within a single population that would have been identified as a potential homolog and removed.

A high degree of colinearity was observed between the consensus map and the physical positions along the 'Golden Delicious' pseudo-chromosomes. Large genetic

gaps were observed in regions of low marker coverage, presumably centromeric and telomeric regions. Over 10% of markers mapped to linkage groups other than the corresponding pseudo-chromosome. These markers should be evaluated for known homology in the *Malus* × *domestica* genome (specifically, known genome duplications and possible misalignments of contigs in the development of the 'Golden Delicious' genome sequence). Colinearity supports the physical ordering of markers, and strengthens the development of meaningful haplotypes that represent true chromosome position. Markers that do not align may result in haplotypes that are a mosaic of different chromosome segments.

The consensus 'Honeycrisp' linkage map was developed from three progeny populations and consists of 1091 SNP markers distributed across the apple genome. These markers were developed from exonic regions from the 'Golden Delicious' genome sequence which adds to their utility in predicting function in marker-locus-trait associations (Chagné et al. 2012). More importantly, these markers are informative in an elite cultivar that is being utilized in breeding programs worldwide for its superb fruit quality traits. QTL analysis in 'Honeycrisp' will focus on identifying the haplotypes associated with crispness, firmness, and juiciness, but will also focus on identifying deleterious associations with postharvest disorders such as soft scald, internal browning, and bitter pit, to which 'Honeycrisp' is prone. Unlike other linkage maps that were developed using bi-parental mapping populations for mapping traits segregating in the parents, the genetic map presented here has enhanced utility and is relevant to many breeders. The clustering of SNPs at single loci due to low recombination and/or as an

artifact of the array design offers additional utility in determining functional haplotypes in QTL analysis of diverse pedigreed germplasm.

Description of Supplementary Materials

Table S1: Table S1 contains the 'Honeycrisp' parental maps from the 'Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark' populations. Marker names are abbreviated and corresponding full names can be found in Table S2.

Table S2: Table S2 contains the consensus 'Honeycrisp' parental map. Full SNP marker names are included from both the IRSC 8k array as well as NCBI dbSNP accession names. 'Golden Delicious' pseudo-chromosome and physical map positions are aligned to the linkage map and links to GDR Gbrowse are provided. Flanking sequence and SNP type are also included.

Table 3.1. Number and percentage of heterozygous markers of the non-'Honeycrisp' parent for its corresponding parental map and in the consensus map for three mapping populations ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark'). 'Honeycrisp' is heterozygous at all mapped loci.

Parent	Heterozygous Markers in Parent	Markers in Parental Map	Proportion of Parental Map (%)	Proportion of Consensus Map (%) (1091 markers)
Gala	448	1042	43.0	41.1
MN1764	359	1041	34.5	32.9
Monark	493	1018	48.4	45.2

Table 3.2. Details from the genetic linkage maps of three 'Honeycrisp' parental maps from three full-sib populations ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark'). Number of markers per linkage group, map size (cM), density, and largest gap are given. Details from the consensus map constructed from the integration of the three 'Honeycrisp' parental maps are also shown in bold.

		LG1	LG2	LG3	LG4	LG5	LG6	LG7	LG8	LG9
Number of Markers	Gala	55	77	48	85	82	51	24	56	90
	MN1764	54	80	50	88	82	51	23	55	84
	Monark	54	77	49	88	69	49	24	51	87
	Consensus	**56**	**83**	**53**	**90**	**83**	**52**	**26**	**57**	**91**
Size (cM)	Gala	57.6	53.95	71.32	50.36	83.08	66.91	66.47	45.98	40.18
	MN1764	50.99	74.21	134.16	125.03	94.46	64.73	73.84	37.32	56.76
	Monark	63.53	51.35	84.02	51.86	76.54	101.9	94.16	51.16	59.37
	Consensus	**71.34**	**78.86**	**112.4**	**84.11**	**108.66**	**78.39**	**89.14**	**61.58**	**72.23**
Average marker distance (cM)	Gala	1.05	0.7	1.49	0.59	1.01	1.31	2.77	0.82	0.45
	MN1764	0.94	0.93	2.68	1.42	1.15	1.27	3.21	0.68	0.68
	Monark	1.18	0.67	1.71	0.59	1.11	2.08	3.92	1	0.68
	Consensus	**1.27**	**0.95**	**2.12**	**0.93**	**1.31**	**1.51**	**3.43**	**1.08**	**0.79**
Maximum gap size (cM)	Gala	11.66	7.78	13.54	7.77	11.66	9.68	31.18	5.94	5.13
	MN1764	8.76	7.71	13.21	7.7	13.21	12.43	38.63	8.76	10.94
	Monark	12.57	8.04	25.14	61.24	14.2	41.96	33.15	11	8.02
	Consensus	**10.87**	**6.88**	**12.24**	**12.92**	**12.39**	**9.48**	**34.21**	**6.33**	**7.91**

Table 3.2 continued (2 of 2)

		LG10	LG11	LG12	LG13	LG14	LG15	LG16	LG17	Total
Number of Markers	Gala	66	59	50	48	42	84	57	68	1042
	MN1764	67	62	51	50	38	84	55	67	1041
	Monark	68	60	51	48	38	85	55	65	1018
	Consensus	**72**	**64**	**52**	**51**	**44**	**91**	**58**	**68**	**1091**
Size (cM)	Gala	63.71	51.61	58.51	104.7	65.63	99.83	54.64	63.07	1097.55
	MN1764	90.56	84.22	63.48	65.05	56.19	134.47	77.85	66.98	1350.29
	Monark	81.72	83.35	60.34	66.34	64.07	89.72	63.11	197.66	1340.2
	Consensus	**91.54**	**97.18**	**75.51**	**97.6**	**75.95**	**130.48**	**74.12**	**82.63**	**1481.72**
Average marker distance (cM)	Gala	0.97	0.87	1.17	2.18	1.56	1.19	0.96	0.93	1.05
	MN1764	1.35	1.36	1.24	1.3	1.48	1.6	1.42	1	1.3
	Monark	1.2	1.39	1.18	1.38	1.69	1.06	1.15	3.04	1.32
	Consensus	**1.27**	**1.52**	**1.45**	**1.91**	**1.73**	**1.43**	**1.28**	**1.22**	**1.36**
Maximum gap size (cM)	Gala	15.87	9.68	8.72	53.36	10.66	16.98	11.66	6.85	
	MN1764	14.38	16.82	12.06	12.06	10.94	20.72	16.82	15.59	
	Monark	14.12	15.85	9.49	14.2	9.49	11	6.59	129.64	
	Consensus	**13.34**	**23.54**	**10.09**	**22.26**	**8.68**	**16.77**	**12.19**	**5.82**	

Figure 3.1. Work flow describing the mapping process including the number of SNP markers retained at each stage.

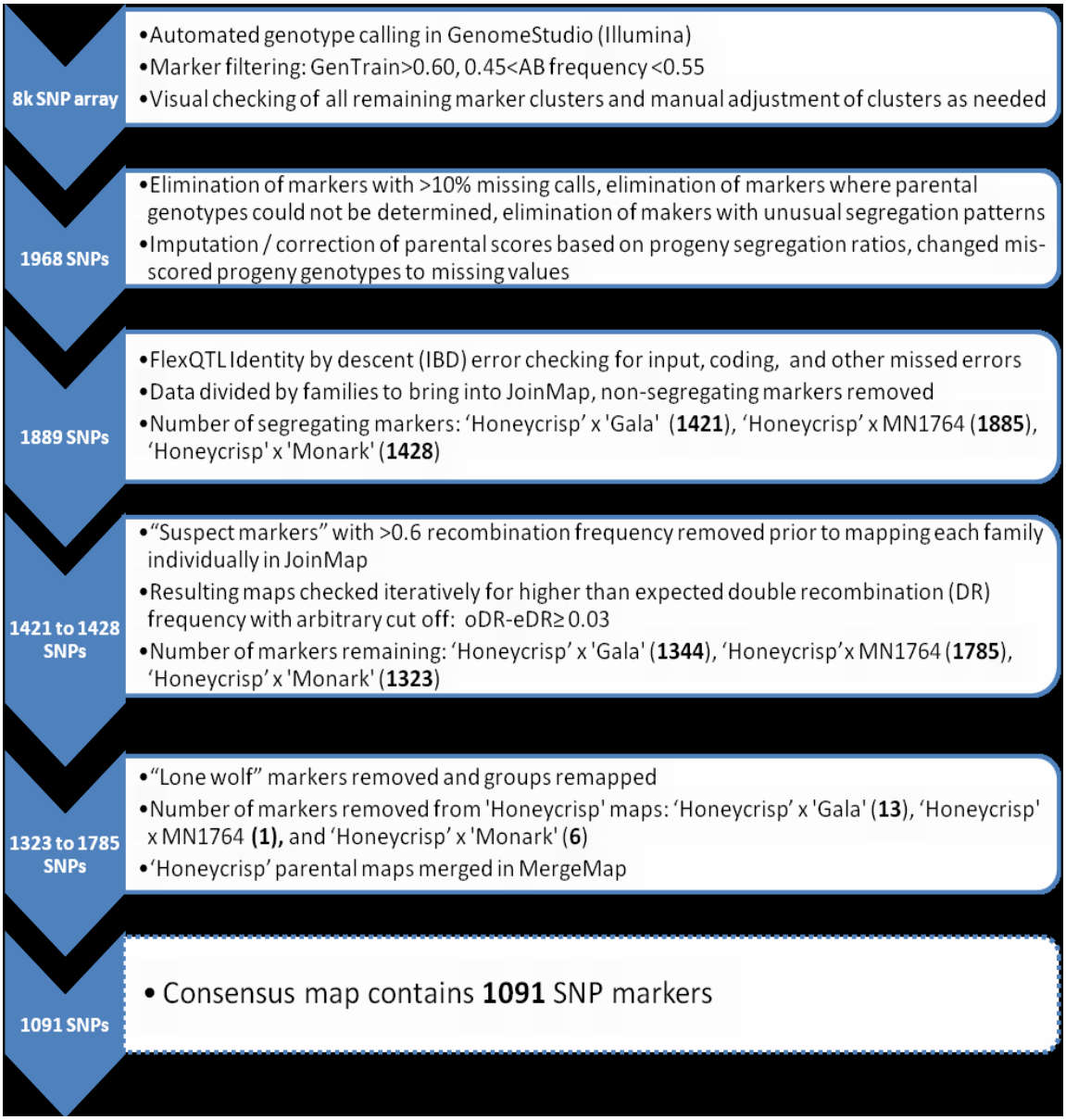


Figure 3.2. Homozygosity plot indicating polymorphism in the parents from the three mapping populations (‘Honeycrisp’ × ‘Gala’, ‘Honeycrisp’ × MN1764, and ‘Honeycrisp’ × ‘Monark’) plotted on the consensus map (x-axis). ‘Honeycrisp’ is heterozygous at all loci. Multiple open circles at a locus indicate more than one SNP marker mapped to that locus for the given parent.

Figure 3.2 continued (2 of 2)

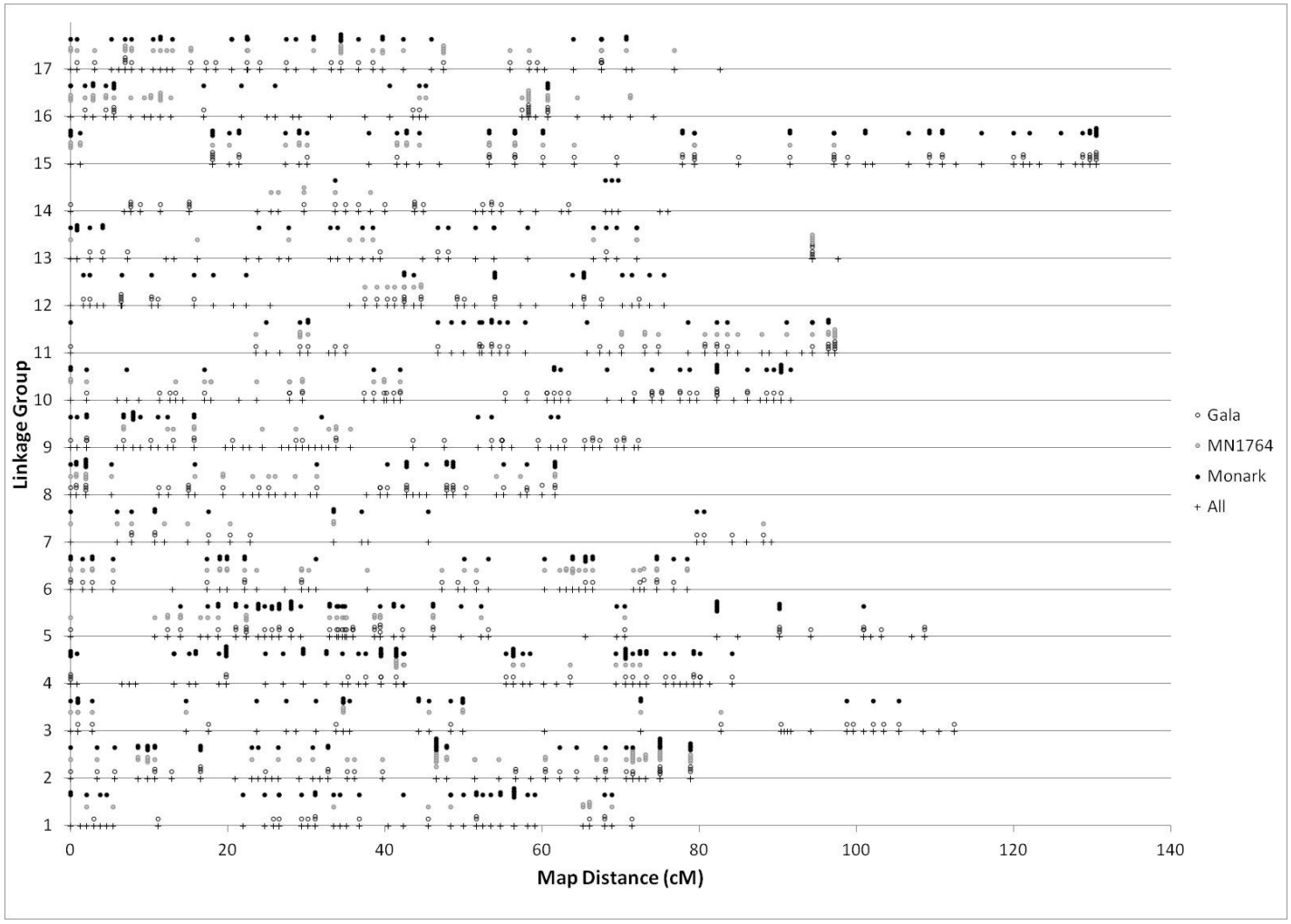


Figure 3.3. Three ‘Honeycrisp’ parental maps (‘Honeycrisp’ × ‘Gala’, ‘Honeycrisp’ × MN1764, and ‘Honeycrisp’ × ‘Monark’) utilized in consensus map construction. Lines between linkage groups show homology between maps within that linkage group. Scale is in cM.

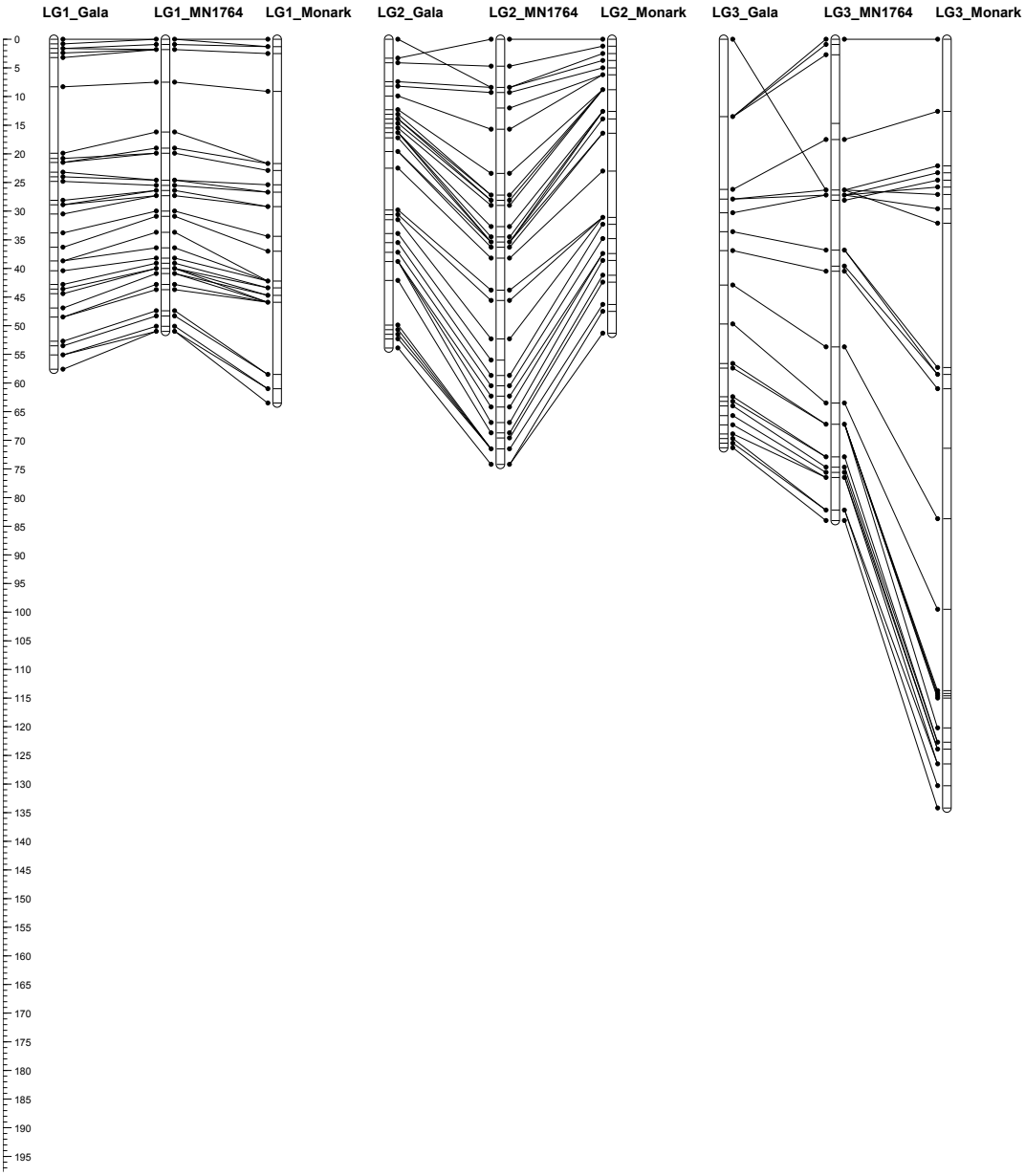
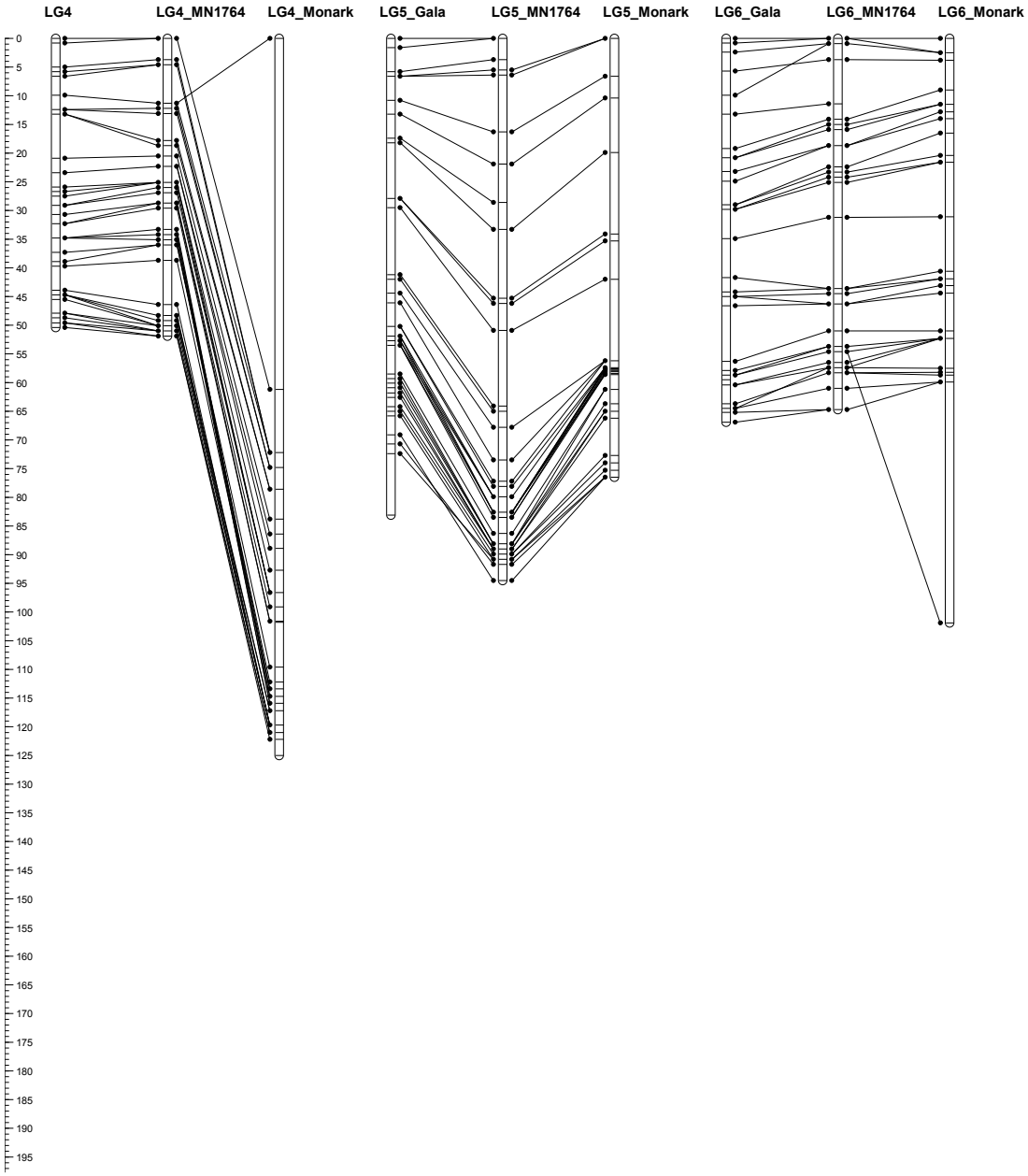


Figure 3.3 continued (2 of 6)



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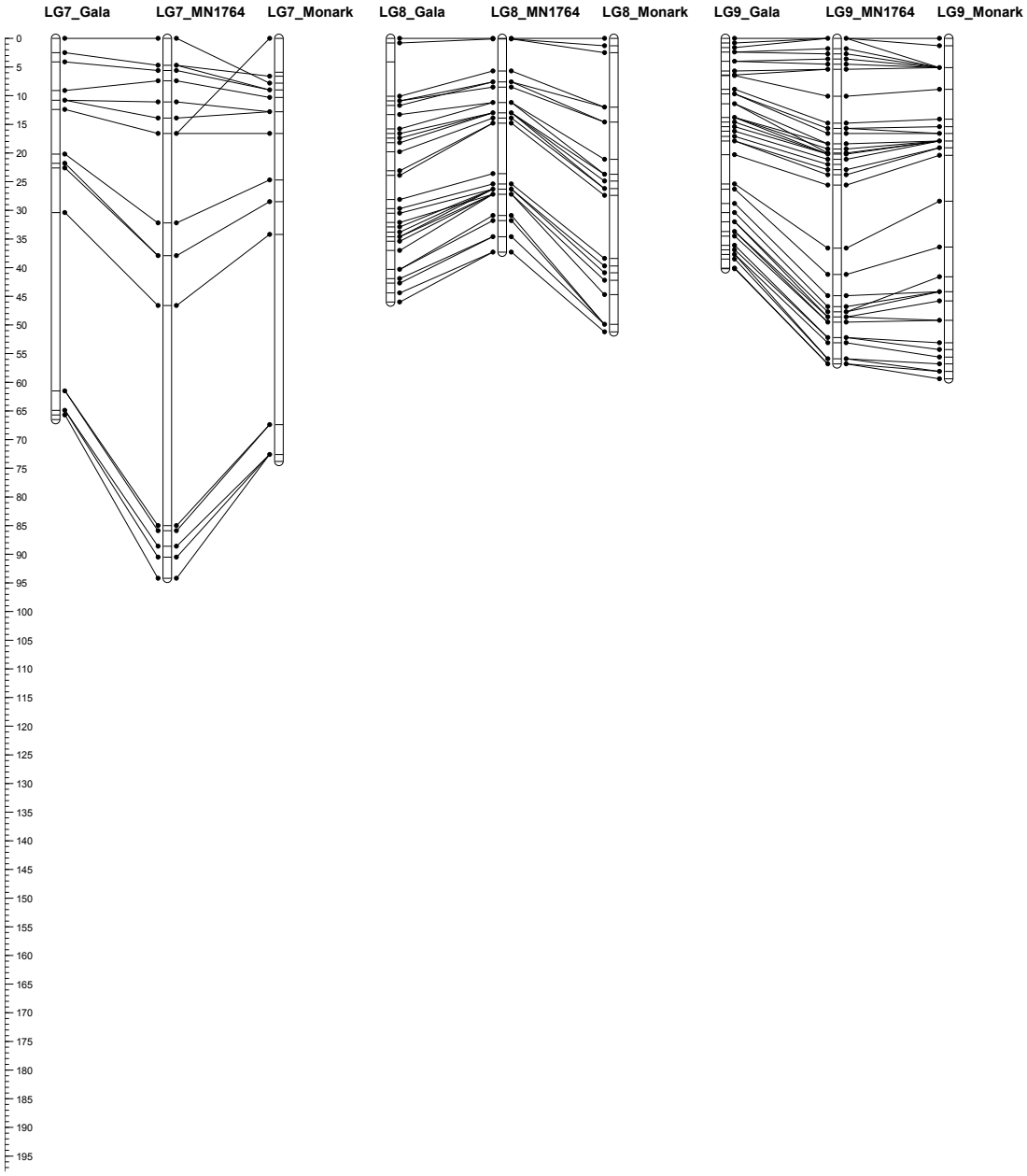
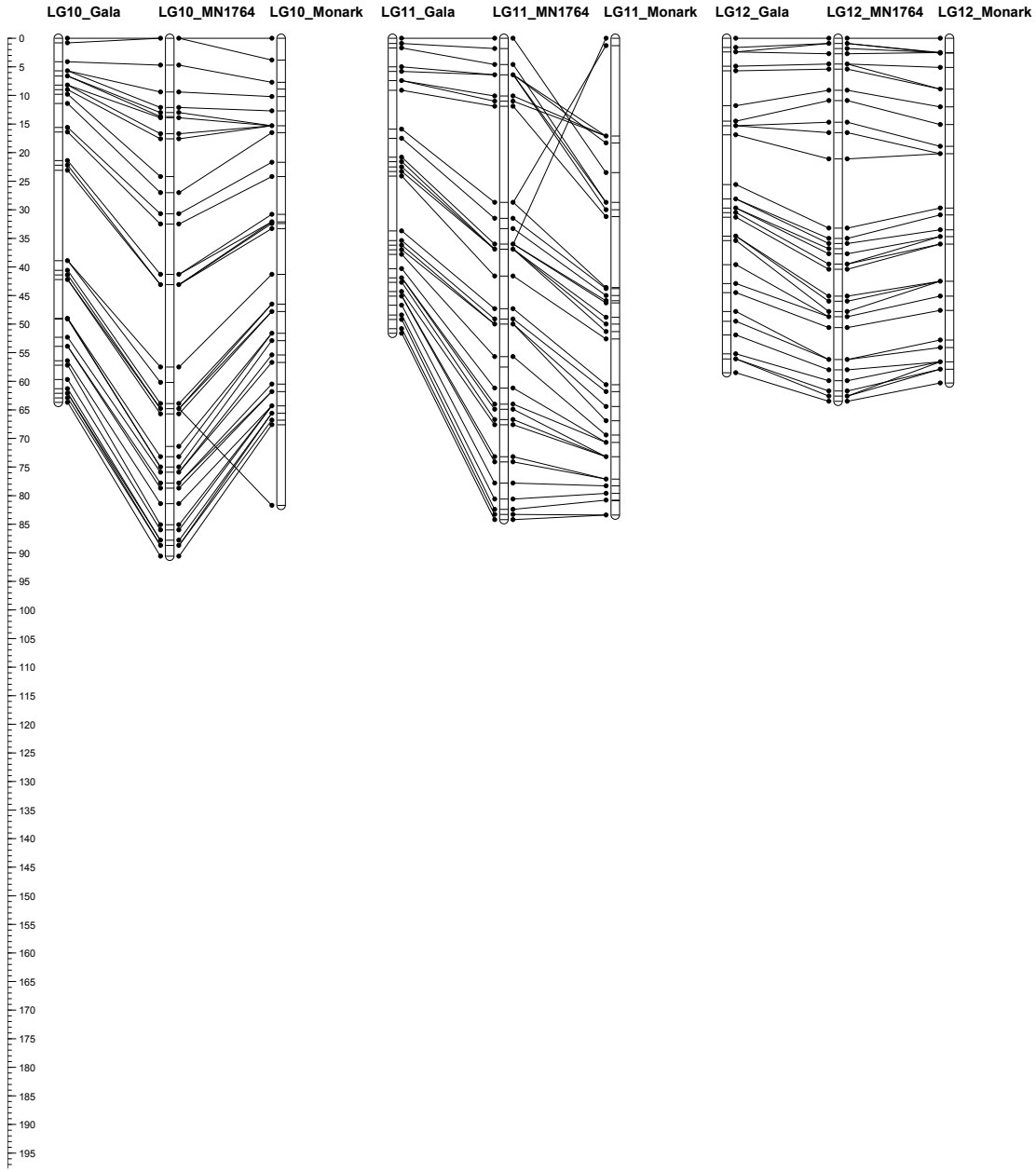


Figure 3.3 continued (4 of 6)



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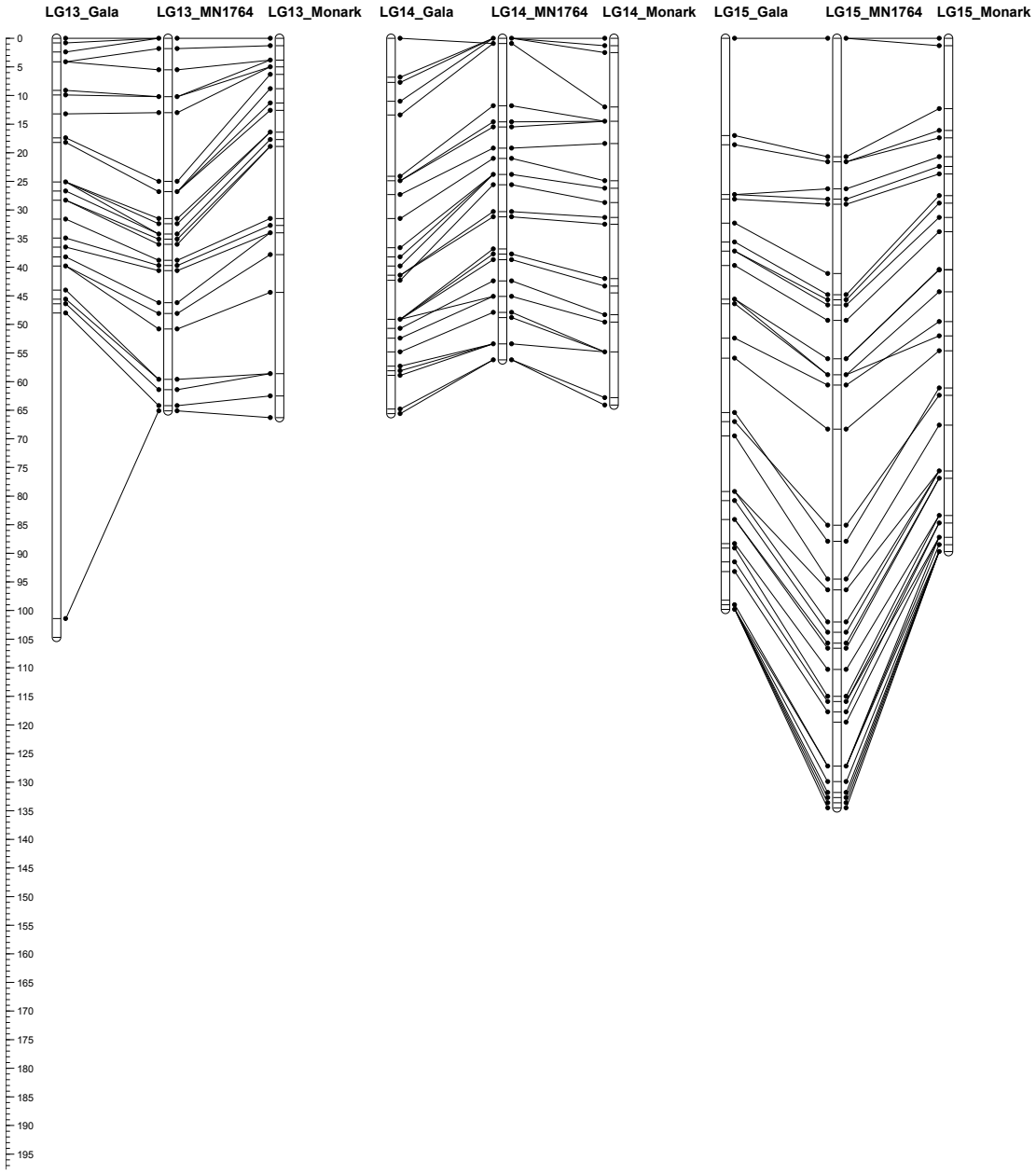


Figure 3.3 continued (6 of 6)

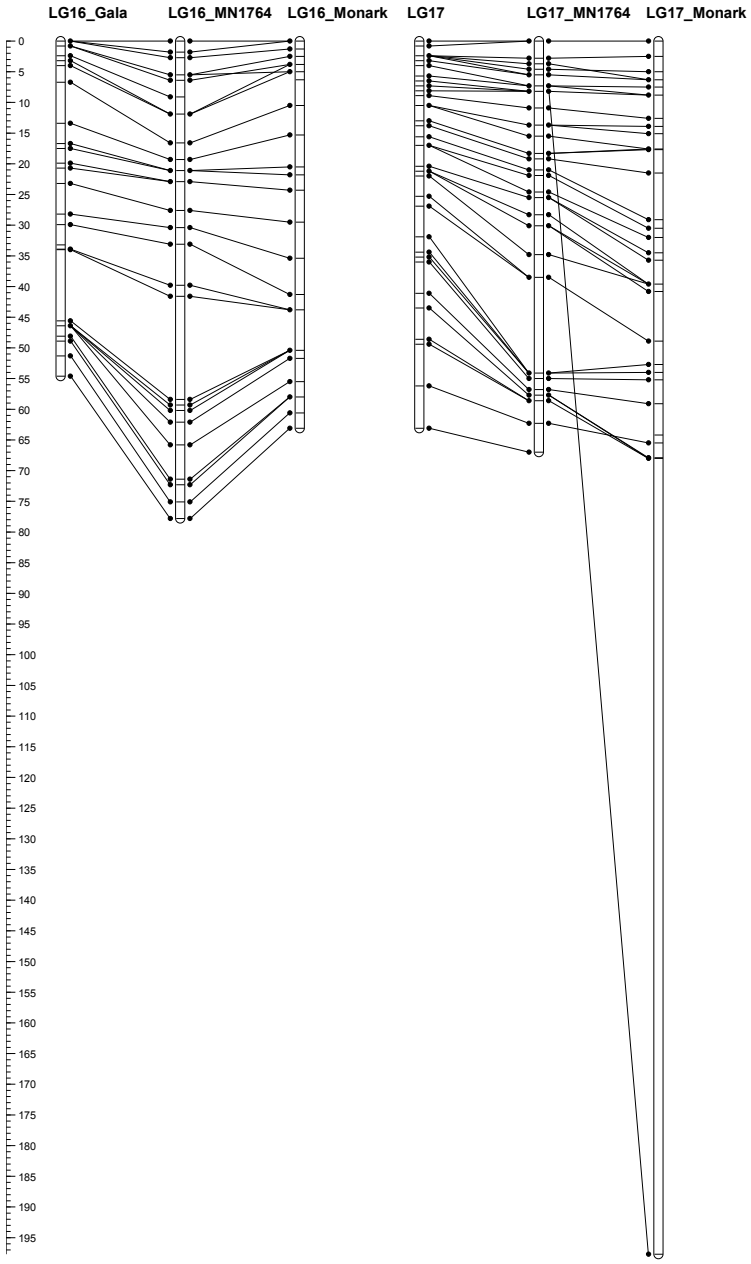


Figure 3.4. Consensus 'Honeycrisp' linkage map constructed from three 'Honeycrisp' parental maps ('Honeycrisp' × 'Gala', 'Honeycrisp' × MN1764, and 'Honeycrisp' × 'Monark'). Markers shown in bold and italic were not common to all three parental maps. Scale is in cM.

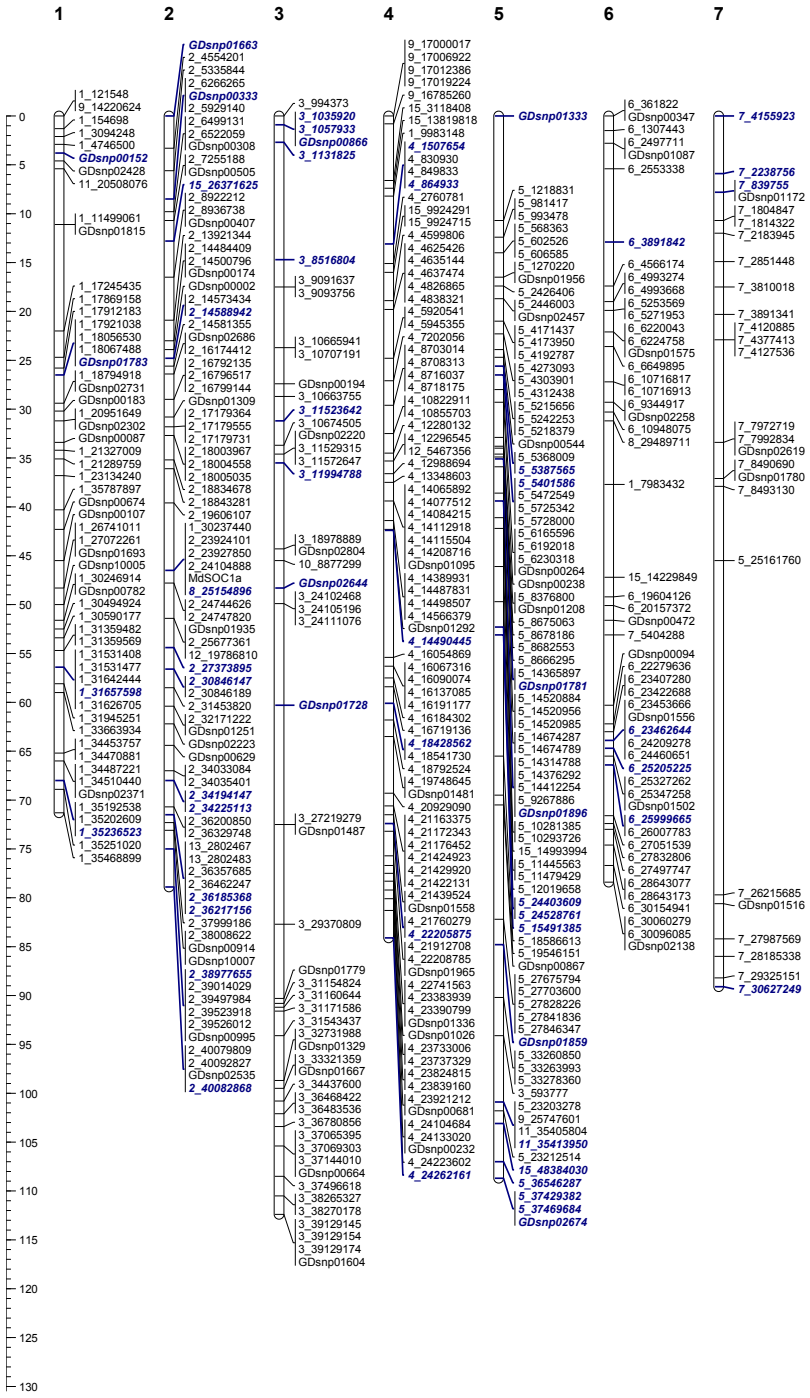


Figure 3.4 continued (2 of 3)

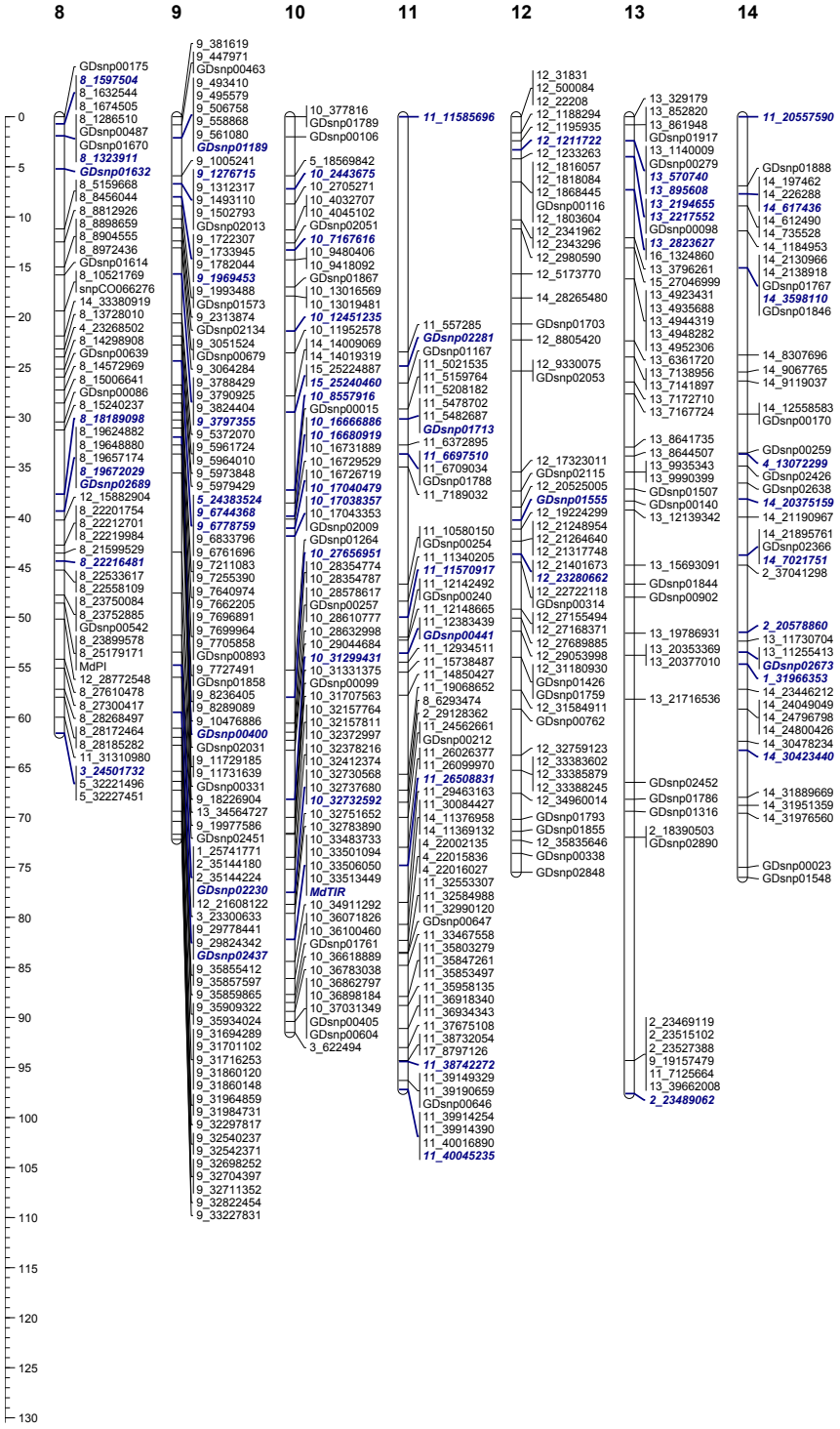


Figure 3.4 continued (3 of 3)

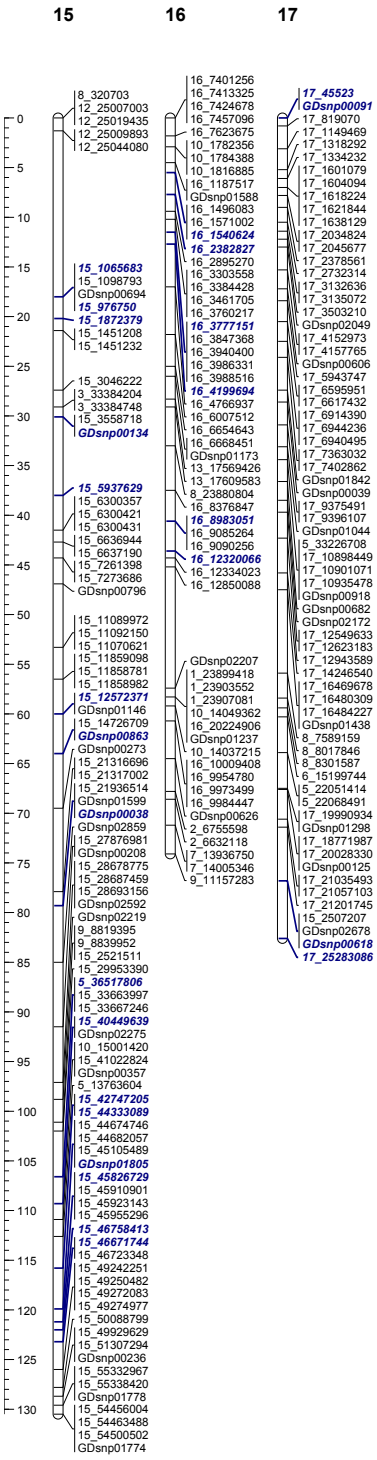


Figure 3.5. Venn diagram showing the number of markers shared in the 'Honeycrisp' consensus map (1091 total SNP markers) and those unique to each population.

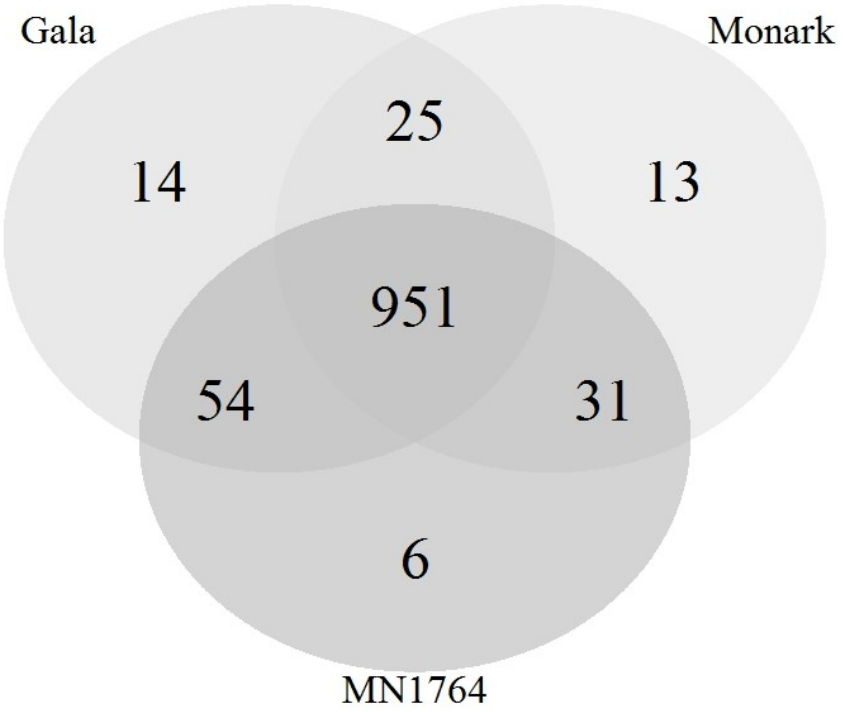


Figure 3.6. Comparison of 'Honeycrisp' consensus map to physical position on 'Golden Delicious' genome sequence for each of the 17 linkage groups. Each plot directly compares the linkage group (LG1- LG17) to the pseudo-chromosome (1-17) available in the Genome Database for Rosacease (www.rosaceae.org). Markers showing segregation distortion (P-value 0.005) are indicated as follows: open circles (○) no significant distortion in any of the three families, gray filled circles (●) significant distortion in one family, and black filled circles (●) significant distortion in two families. No markers in the consensus 'Honeycrisp' linkage maps showed significant segregation distortion in all three of the mapping populations.

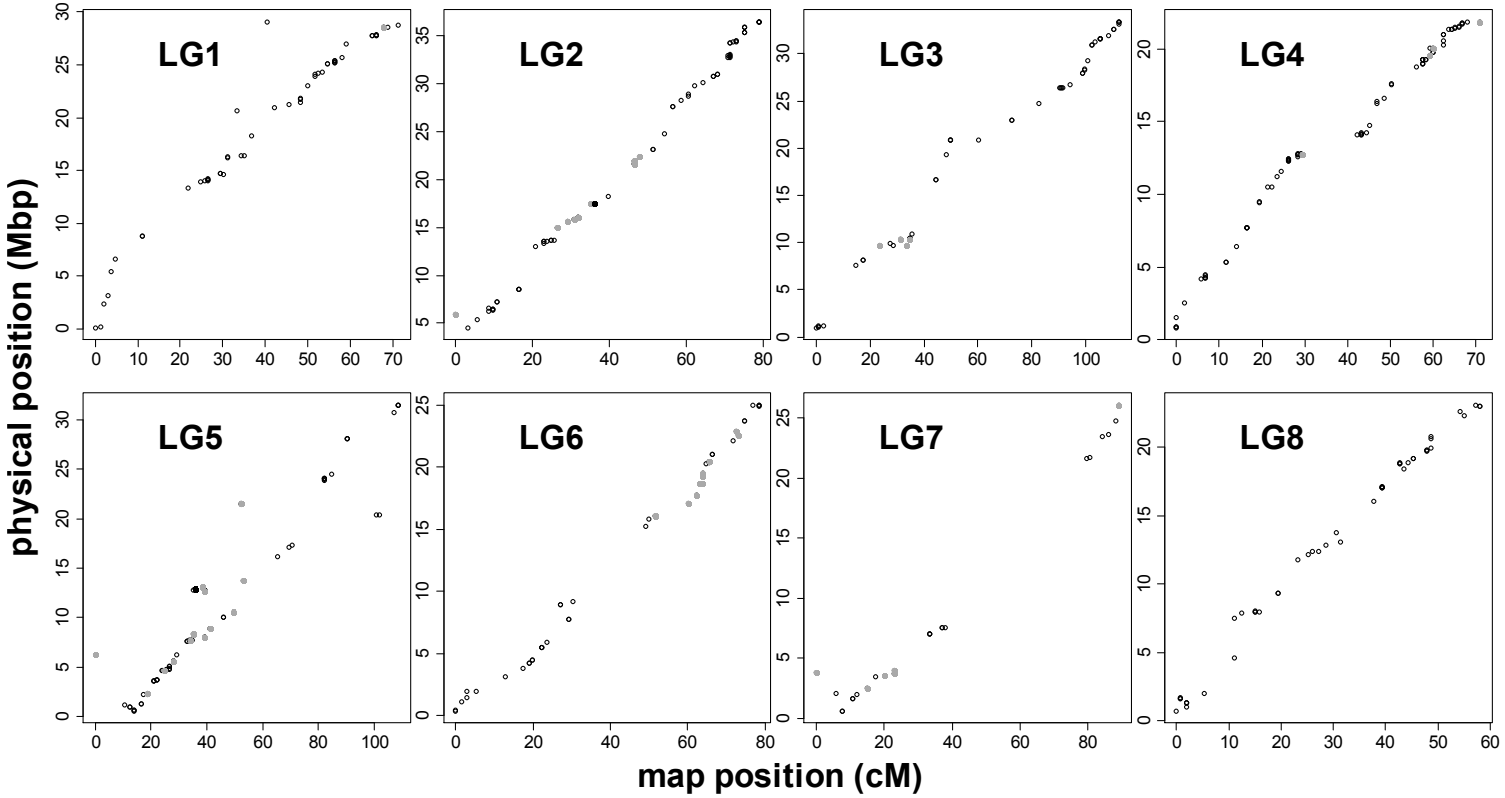
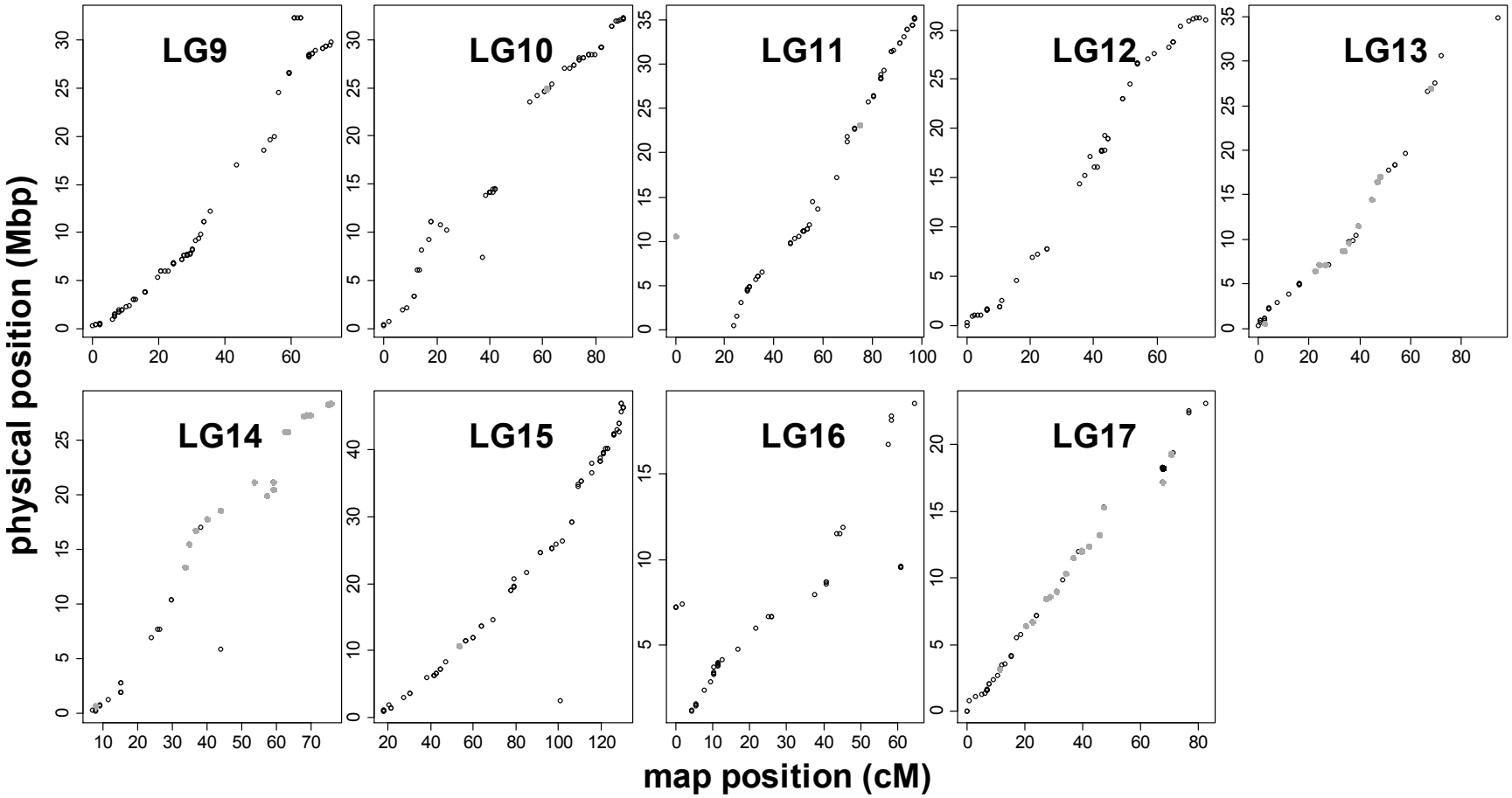


Figure 3.6 continued (2 of 2)



CHAPTER FOUR: QUANTITATIVE TRAIT LOCI AND FUNCTIONAL HAPLOTYPES FOR FRUIT TEXTURE TRAITS IN 'HONEYCRISP' PROGENY

Introduction

Breeding advances in apple (*Malus* × *domestica*, Borkh.) have been limited due to long juvenility, self-incompatibility, and high heterozygosity. Apple seedlings can take upwards of six years to reach fruiting maturity, greatly delaying, relative to non-tree crops, the breeder's chance to directly evaluate fruit quality traits in breeding progenies. Therefore, breeding in tree crops, such as apple, could be greatly advanced by marker-assisted selection (MAS). MAS of seedlings (marker-assisted seedling selection, MASS) allows for selection upon traits not yet expressed such as fruit quality and certain disease resistances, speeding breeding progress toward better-adapted cultivars exhibiting high fruit quality by enhancing the likelihood that planted seedlings will prove useful. MASS would be cost-efficient when heritability is low, the trait is not easily phenotyped in young trees, and when the costs of carrying additional trees to maturity substantially outweighs the cost of marker characterization (Luby and Shaw, 2000). Additionally, breeders might use marker-assisted parent selection (MAPS) to make better-informed crosses by choosing breeding parents most likely to confer favorable allele combinations upon the progeny. MAPS only requires genotyping of prospective parent trees, which is less costly than genotypic analysis of an entire progeny and is thus an ideal starting point for introducing MAS into a breeding program. Recent advances make DNA-informed breeding in apple a reality.

Velasco et al. (2010) published a high-density genome sequence of the 'Golden Delicious' genome, bringing apple into the realm of modern genetics applications. With the sequenced genome, spacing single nucleotide polymorphism (SNP) markers with confidence across informative parts of the apple genome became feasible. Following publication of the genome, the International RosBREED SNP Consortium (IRSC) apple Illumina Infinium® II 8K SNP array v1 was developed (Chagné et al., 2012). SNPs were detected through sequencing of 27 additional apple cultivars representative of breeding germplasm used the world over. The apple Infinium® II array v1 consists of 7,867 SNPs in 1,355 clusters (Chagné et al., 2012). Clusters of four to ten SNPs, spaced about 10 cM apart, were chosen to enable the identification of functional haplotypes when narrow genomic regions of interest can be determined.

Apple's ancestral genome-wide duplication (GWD) was confirmed with the genome-sequencing project (Velasco et al., 2010) and the high collinearity observed among entire chromosomes and large chromosome segments made contig assembly and assigning final physical map order challenging. Indeed, using the IRSC 8K SNP array to construct an apple rootstock progeny linkage map, Antanaviciute et al. (2012) encountered evidence of GWD. Evidence of GWD was apparent both as inability to assign genotypic state to some markers that likely annealed to paralogous loci or a locus having copy number variation and in finding that 13.7% of mapped markers did not map to linkage groups corresponding to the pseudo-chromosome assignment made by Velasco et al. (2010).

Due to the aforementioned difficulties in determining marker physical positions in apple, linkage maps specific to the germplasm of interest are especially useful in determining marker-trait associations. Moreover, this may avert issues in which the individuals of interest have chromosomal inversions, translocations, or deletions with respect to the sequenced genome. Linkage maps in outcrossing species such as apple are developed using the two-way pseudo-testcross method (Grattapaglia and Sederoff, 1994), in which a single map is calculated for each parent consisting of only markers segregating in that parent.

In tree species, quantitative trait loci (QTL) have most commonly been identified in single full-sibling populations (Costa et al., 2010b; Kenis et al., 2008; King et al., 2000; and Liebard et al., 2003), with validation in additional populations. An alternative to finding and comparing QTL in several full-sib families is pedigree genotyping, examining several generations of related populations at once (van de Weg et al., 2006). Bink et al. (2008) describe three advantages of studying inter-related families with multiple founders, as opposed to individuals sharing the same parents: improved ability to detect valuable QTL alleles, relevance of detected QTL in the breeding population, and cost effectiveness. The cost of genotypic characterization of individuals with select markers is becoming increasingly practical in comparison to that of labor and cost to phenotype the same individuals, especially for traits that are not expressed annually or until tree maturity.

Using pedigree-based analysis, FlexQTL™, a software package from Wageningen UR, employs Bayesian analysis with Markov Chain Monte Carlo (MCMC)

algorithms to detect QTL with phenotypic trait and genotypic marker data (Bink et al., 2008). The European Union High-Quality Disease Resistant Apples for a Sustainable Agriculture (EU HiDRAS) project was developed as a trial for pedigree genotyping in which selections in several related families and progeny were concurrently assessed for QTL (Antofie et al., 2007). Bink et al. (2008) demonstrated the utility of FlexQTL™ on a subset population from HiDRAS consisting of 604 individuals, comprising 13 full-sib populations, descending from 15 founder cultivars.

Fruit quality traits are of special interest to apple breeders and are ideal candidates for MAS, as fruit traits cannot be evaluated until trees reach maturity. Characteristics of interest in dessert apples are external appearance, eating experience, and the maintenance of such during storage (Janick et al., 1996). The cultivar Honeycrisp displays exceptional fruit quality, and has become an important parent in the University of Minnesota's apple breeding program, as well as in others in the United States and Canada. 'Honeycrisp', when crossed with another cultivar, offers the potential to produce progeny carrying the causal genes of the texture phenomenon. Ideally, breeders would be able to use MAS to incorporate favorable texture alleles from 'Honeycrisp' into genetic backgrounds showing additional disease resistances, tree vigor, and resistance to storage disorders as well as better adaptability to other apple growing regions.

This study takes advantage of new technologies to re-assess a set of five 'Honeycrisp'-derived families described by McKay et al. (2011) in which QTL for apple texture traits were identified (McKay, 2010). Originally, all individuals were genotyped with a set of Diversity Arrays Technology (DArT) markers (Schouten et al., 2012) and a

small set of simple sequence repeat (SSR) markers. Linkage groups were constructed for each of the five families, and QTL for texture traits characterized over three years were found in each of the five families independently (McKay, 2010). Developments in the areas of molecular genetics and computational genetics now allow us to consider texture phenotype data from these five populations simultaneously to identify and validate QTL via pedigree-based analysis of marker-genotype data from the IRSC 8k SNP array.

Materials and Methods

Plant material Phenotypic texture data were obtained from five full-sib families as described by McKay et al. (2011). Each of the five families has 'Honeycrisp' as one parent and the other parent is MN1764, MN1702, 'Monark', 'Jonafree', or PI279323, an accession of 'Pitmaston Pineapple' (hereafter referred to as Pitmaston). Crosses were made between 1992 and 1998 (McKay, 2010) and all trees in this study were planted on the Budagovsky 9 dwarfing rootstock at the University of Minnesota's Horticultural Research Center in Chaska, MN.

Phenotypic evaluation and genotypic value prediction McKay et al. (2011) employed sensory panelists to evaluate fruit within one week of harvest for the sensory traits crispness, firmness, and juiciness. For complete methods, see McKay (2010) and McKay et al. (2011). In brief, panelists were presented with apple wedges and directed to determine crispness during biting and firmness during chewing. Not all panelists received

all fruit, but each fruit was presented to more than one panelist. Panelists recorded their scores for the texture traits on 16 cm generalized Labeled Magnitude Scales, which were later measured. The measured lengths were divided by 16 to get sensory trait scores between 0.00 and 1.00. Over the three years of the study, 23 panelists were involved and nine were constant across all three years. The sensory traits were adjusted to remove panelist effects and best linear, unbiased prediction (BLUP) was used to calculate phenotypic trait values for each genotype-by-year combination. Pearson's correlations between firmness and crispness were calculated for each family for each year and counts of overlaps in individuals with phenotypic data across years were parsed out. Both are useful in considering differences and similarities in QTL placement between years and the predictive value of identified QTL within families across years.

Genotypic evaluation and linkage map construction All individuals in this study were genotyped with the International RosBREED SNP Consortium (IRSC) apple 8K SNP array v1 (Chagné et al. 2012) after leaf collection, DNA extraction and DNA quantification as described in Chapter 3. Linkage maps were constructed using the two largest families, 'Honeycrisp' × 'Monark' (n=81) and 'Honeycrisp' × MN1764 (n=112) and an additional, independent family 'Honeycrisp' × 'Gala Twin Bee' (n=125) that had been created for a scab resistance screening study. The 'Honeycrisp' parental maps from each of these families were merged into a consensus 'Honeycrisp' map, in which all markers were heterozygous in 'Honeycrisp', as described in Chapter 3.

QTL detection and 'Honeycrisp' haplotype determination QTL were determined in pedigree-based analyses using all five progeny sets and six parents. Analyses were conducted with FlexQTL™ versions 0.099102 and 0.099103. 'Keepsake', a parent of 'Honeycrisp' (Cabe et al., 2005) and its parents, 'Frostbite' and 'Northern Spy', were also included in the analyses. Phenotypic data were only available for individuals belonging to the progeny sets, but inclusion of genotypic data from grandparental individuals allows for visualization of identity by descent (IBD) in Pedimap© 1.2 (Voorrips, 2011).

Multiple runs of each trait-year were conducted until an appropriate Markov chain length was determined which would result in an effective chain size of about 100 for the mean. Previous analyses of these data using the Clark et al. (2013) linkage map yielded no strong evidence for QTL with 2005 data (results not shown). Therefore, QTL analyses were conducted for only 2006 and 2007 data since each conclusive whole-genome QTL analysis with a dense linkage map can require a run time of more than a week on a desktop computer. Prior assumptions were for one QTL and a maximum of five to 20 QTL.

SNP haplotypes of the parents were assigned manually in Microsoft Excel for each of the indentified QTL regions with strong or decisive evidence, and the progeny individuals were scored for the 'Honeycrisp' allele carried at each region of interest. Loci at which individuals had missing marker scores, inhibiting haplotype assignment, were marked as missing data, as were the haplotype intervals in which an individual had a crossover event.

'Honeycrisp' haplotype effects Three-way ANOVAs for each trait in year combination were constructed for each family with regions of interest (named for their linkage group position: LG03, LG10, and LG16) as factors. Analyses were done with the statistical software R using the aov function (R core team, 2013). Because the data are unbalanced due to crossover events and haplotypes that could not be positively determined being scored as missing data, the order of the factors in some instances impacted significance of a specific factor, multiple factors, or interactions. To develop a more accurate impression, all analyses were calculated in each of the six possible orders. For factors significant p=0.1 in one or more of the ANOVA for a trait-year, the average means square (MS) proportion of that factor, across all six ANOVA, was calculated.

Tukey's highly significant difference tests were used to determine the trait-increasing 'Honeycrisp' haplotype at each of the significant loci, using a p-value of 0.05. Since one parent of 'Honeycrisp' is unknown (Cabe et al., 2005), IBD analyses were conducted with FlexQTL™ and results were visualized in Pedimap© v1.2 to determine the most likely parental origin of the increasing haplotype at each 'Honeycrisp' locus of interest.

Results

Venn diagrams were constructed for each family, comparing numbers of individuals phenotyped in each year (Figure 4.1). The MN1764 family was the most consistently available for phenotyping, having 20 evaluated individuals all three years.

The least overlap in evaluated individuals across families occurred between 2006 and 2007. Pearson's correlations between crispness and firmness ranged from 0.50 (2005, MN1702 progeny) to 0.92 (2005, 'Monark' progeny; Table 4.1).

QTL Several QTL for both firmness and crispness were identified by FlexQTL™ from the 2006 data (Table 4.3). FlexQTL™ output reports a metric of likelihood for genomic and linkage group QTL placement, equal to two times the natural log of Bayes Factors (BF). On this scale, a 2ln(BF) of 2 to 5 is positive evidence, 5 to 10 is strong evidence and greater than 10 is decisive evidence of a QTL (Bink et al., 2008; Kass and Rafterly, 1995). QTL with decisive evidence were on LG03 and LG10, appearing to cover the same region for both traits in 2006. Additionally, a QTL having strong evidence for firmness was found on LG16 for 2006 data. Analysis of 2006 crispness data also yielded QTL with positive evidence on LG07 and LG15. Analysis of 2007 firmness data yielded strong evidence for the previously identified region on LG03, strong evidence for a QTL on LG16, and positive evidence for a QTL on LG14. Only QTL with decisive evidenced were considered in further analyses. Differences between QTL analyses of 2006 firmness and 2007 firmness in the degree of convergence of the iterative FlexQTL™ process and difference of placement of QTL peaks is depicted in Figure 4.2. A summarization of FlexQTL™ analyses parameters and results is given in Table 4.3.

Haplotypes The two 'Honeycrisp' haplotypes were determined for the two QTL determined for 2006 crispness and firmness, as well as a third QTL that was determined

for 2006 firmness (Figure 4.3). Progeny were only scored to determine which 'Honeycrisp' haplotypes (i.e. 'Honeycrisp' A or B at each locus) were present.

The region of interest on LG03 encompassed seven markers spanning 8.74 cM of the 'Honeycrisp' consensus map, from marker 3_31154824 to marker 3_33321359. Due to heterozygosity of MN1702 at 3_33321359, haplotypes for the MN1702 progeny were assigned in the region spanning from 3_31154824 to GDsnp01329, the genetic distance of which is 7.94cM.

The region of interest on LG10 spanned 19.66cM from marker 10_1245123510 to 10_16726719 and contained 14 SNP markers of the consensus 'Honeycrisp' linkage map. Due to heterozygosity of 'Pitmaston' and 'Jonafree' at the ends of this region, progeny in these families were assigned haplotypes in a slightly smaller region (10_11952578 to 10_16726719, 17.49cM and 14_14019319 to 10_17040479, 13.23cM, respectively). MN1702 was highly heterozygous in the region of interest on LG10 and therefore haplotypes were assigned to the progeny for the 31.64cM region between 10_11952578 and GDsnp01264.

The 11.44cM haplotyped region associated with the QTL on LG16 consisted of 16 SNP markers and was defined by markers 16_1540624 and 16_4766937. Due to heterozygosity of the non-'Honeycrisp' parents, the 'Monark' and 'Jonafree' progenies were assigned haplotypes for the region from 16_2382827 to 16_4199694 (5.05cM). Likewise, the 'Pitmaston' progeny were assigned haplotypes for the region from 16_2895270 to 16_4766937 (7.55cM) and MN1702 progeny were assigned haplotypes for the region 16_1540624 to 16_6654643 spanning 19.51cM.

The percent of individuals with a missing score, either due to the rare case of a missing block of marker score data or a crossover event within the region, was greatest in the region on LG10, the largest region analyzed (Table 4.2; ranging from 11.8% to 17.9% among families). There were four instances in which a haplotype could not be determined. These include one individual at each region in the 'Monark' family and one individual at the LG03 region in the Pitmaston family. The regions of LG03 and LG16 were comparable in frequency of crossover, ranging from 0% (MN1764 progeny) to 8.6% ('Monark' progeny) for LG03 and from 0% ('Jonafree' progeny) to 9.8% (MN1764 progeny) in the LG16 region.

Utility of identified regions in predicting phenotypic values Average proportions of total MS attributed to factors significant at p=0.1 in at least one of the six ANOVA orders were determined within each family for each year-trait instance (Figures 4.4 and 4.5). The 'Honeycrisp' regions identified more often explained variation in firmness than in crispness (26 vs. 13 instances, respectively) but this may be additional evidence, in support of the results of QTL analyses, that crispness is controlled by fewer loci with large effects than firmness. LG03 was significant in more instances than LG16 and LG10 (13, 8 and 7 instances, respectively). The 'Honeycrisp' haplotype on LG10 only explained a significant proportion of crispness in one year, 2006, in the MN1764 progeny, whereas all other main effects were significant in at least three family-trait-year instances.

'Honeycrisp' haplotypes The "A" haplotype had an increasing effect, when significant, at the LG03 and the LG10 loci. At the LG16 locus, the "B" haplotype was increasing when there was a significant difference between haplotypes. Each of the two-way interactions were significant in some instances and contributed as much as 60% and 68% of the total MS for 2005 firmness and 2007 firmness (LG03:LG10 and LG03:LG16) in the Monark population. The three-way interaction was never significant. The IBD functionality of the FlexQTL™ software was used to develop Pedimap© input to visualize the parental origin of 'Honeycrisp' haplotypes (Figure 4.6). The 'Honeycrisp' "A" haplotype on LG03 appears to come via 'Keepsake' from 'Frostbite'. The 'Honeycrisp' haplotype conferring higher trait values at LG10 comes from the unknown parent of 'Honeycrisp'. The 'Honeycrisp' "B" haplotype at the LG16 region comes from 'Northern Spy' by way of 'Keepsake'. Histograms of trait distributions for individuals having none, one, two, or all three of the trait-increasing 'Honeycrisp' alleles (Figures 4.7 and 4.8) illustrate a trend toward increasing firmness and crispness values as each additional allele of increasing effect at one of the loci moves the distributions to the right.

Discussion

Reports in the literature of QTL for sensory crispness and firmness support the chromosomal placement of marker-trait associations identified in this study of these populations. Comparison of identified regions within linkage groups is difficult because marker types and marker density were unique among studies and linkage group

orientation may not be reliable in early studies. A QTL for texture traits is often reported on LG10 (i.e. King et al., 2000; King et al., 2001; Kennis et al., 2008; and Costa et al., 2010b). Liebhard et al. (2003) reported a QTL for fruit firmness on LG03 accounting for 27% of the observed phenotypic variance. King et al. (2000) reported QTL for sensory crispness and hardness on LG10, and additionally, a QTL of large effect on linkage group 16 explaining 24% of the variance in crispness. With the high correlation between firmness and crispness within years observed in this study, it is not surprising that the region on LG16 detected with FlexQTL™ for firmness has been identified in other studies for crispness. King et al. (2000) define crispness in terms of "crunchy noise when chewing", while other researchers reserve the expression to describe an experience during biting (i.e. Evans et al., 2012). Differences in how sensory panels were trained and how traits are defined may also play a role in differences across studies. Interestingly, King et al. (2001) identified QTL for an instrumental texture measure on LG07 and LG15, which were detected as regions of positive evidence for 2006 crispness in this study but were not pursued in favor of regions on LG03, LG10 and LG16 with much higher 2ln(BF) scores. None of the above studies considered progeny of 'Honeycrisp' or the other parents in this study, yet similar regions were reported as those identified in this study, suggesting that the very unique texture of 'Honeycrisp' arises from a unique allele or copy number variation in genes at these regions responsible for texture variation in all apples or from other, as yet unidentified regions.

McKay (2010) studied QTL for texture traits within these five families using the phenotypic data presented here. He analyzed each family separately rather than using the

pedigree-based approach and used linkage maps with much lower marker density and far fewer codominant markers. McKay (2010) reported no QTL for 2005 crispness on LG03, and QTL on LG03 for firmness in the MN1764 family in 2005 and 2007 data. Mapping QTL for average firmness across the three years, McKay found QTL on LG03 for the 'Monark' and Pitmaston families. On LG10, McKay found no QTL for 2005 data, one for crispness in the 'Monark' family in 2006 and QTL for 2006 firmness in the MN1702 and 'Monark' families. Analysis of the 2007 data indicated a QTL for the MN1764 family explaining 47% of the variation in firmness on LG10. On LG16, McKay found support for QTL for 2007 crispness in the MN1702 family and for 2005 and 2006 firmness in the same family as well as a QTL explaining 36% of the variation for 2005 firmness in the MN1764 family. McKay (2010) reports a QTL for sensory and instrumental texture traits on all but two of apple's 17 linkage groups though none of these marker-trait associations appeared sufficiently robust to use in implementing MAS in the breeding program.

McKay et al. (2011) reported a correlation of 0.85 between crispness and firmness across all families, averaged over years. We report correlations by year and for each family for a better understanding of when crispness and firmness QTL might be expected to collocate. The high correlation between crispness and firmness in the 'Monark' family in 2005 (0.92) is reflected in a significant interaction between LG03 and LG10 for both traits that year, although the MS proportion attributed to each differs in magnitude. The correlation between crispness and firmness in the 'Jonafree' progeny was 0.88 in 2005 and our results showed significance of LG03 for both traits in that year, having a similar

effect size. In both of these instances, a greater proportion of variation was explained for firmness than for crispness. An instance of the opposite pattern can be seen in the MN1702 family, which in 2006 exhibited a correlation of 0.87 but the LG03 region explained more of the variation for crispness than for firmness and was significant for all six ANOVA for crispness and was only supported by three of the six ANOVA orders for firmness. Within-family differences between firmness and crispness in regions to which variance is attributed and portion of variance explained could be useful in determining a scheme to select for one or the other of these typically highly correlated traits. This would need to be attempted in larger progeny sets, with balanced data to accurately determine the effects of interactions between these loci.

For firmness, the regions on LG03 and LG10 were supported in at least one family in all three years and the region on LG16 was supported in 2006 and 2007. For crispness, LG03 was supported in at least one family in all three years, but the region on LG16 was only supported by the 2006 and 2007 data and the region on LG10 only by the 2006 data in one family. This suggests that selection for 'Honeycrisp' haplotypes at the region on LG10 may be less effective in increasing crispness than it would be in increasing firmness. McKay et al. (2011) reported a lower broad-sense heritability estimate for crispness (0.76) than firmness (0.81). However, stacking the three trait-increasing 'Honeycrisp' alleles could be an effective breeding strategy, as the distributions of both texture traits are positively affected by having an increasing quantity of these alleles.

The knowledge that genes associated with fruit firmness in other studies, *Md-PG1* and *Md-AC01* map to LG10 (Costa, et al. 2010b) suggested that one of these may be a candidate genet underlying the QTL effect on LG10 of this study. A blastn search of the *Md-PG* gene (GenBank AF031233.1) placed it at chr10: 18,131,524...18,146,176bp, while the markers of the LG10 haplotyped region reported here are above the *Md-PG* gene, spanning ch10:7,424,669...14,508,549bp. Costa, et al. (2010b) mapped the *Md-AC01* locus below the *Md-PG1* locus. Although the QTL does not include *Md-PG1*, the region could be linked to the identified gene.

Conclusions

This study made use of existing, high-quality phenotypic data that described texture traits in five progeny sets over three years. The progeny were developed as part of routine breeding efforts. Coupling these data with the more recent technologies of the IRSC apple 8K SNP array v1 and pedigree-based QTL analysis, we determined three regions of marker-trait associations that could be targeted for marker development to enable MAS for fruit crispness and firmness in breeding programs using 'Honeycrisp' as a parent. Pedigree-based analysis is robust in that it can be used to combine many sets of related materials, as often exist in breeding programs because the best parents are used frequently. This is an advantage also in that marker-trait associations are determined in genetic backgrounds present and in use in the breeding program.

To enable MAS of the identified regions within a breeding program using 'Honeycrisp' or its progeny as parents, simple sequence repeat markers could be designed around these loci. Markers could be validated using the individuals of this study, those of the RosBREED study discussed in Chapter 2, and additional 'Honeycrisp' progeny already existing in several breeding programs in the U.S. and globally.

Table 4.1 Pearson's correlations between crispness and firmness, by family and year.

	Jonafree	Pitmaston	MN1702	Monark	MN1764
crisp05:firm05	0.88***	0.66***	0.50**	0.92***	0.77***
crisp06:firm06	0.89***	0.86***	0.42*	0.88***	0.87***
crisp07:firm07	0.69***	0.89***	0.69***	0.80***	0.74***

***, **, * significant at $p \leq 0.001$, 0.01, or 0.05 respectively

Table 4.2 Percentage of individuals at each locus interval having a crossover event or missing data (for which the haplotype could not be determined), by family. Map distance is the length of the region, as represented in the consensus 'Honeycrisp' map presented in Chapter 3.

	Map distance (cM)	Jonafree	Pitmaston	MN1702	Monark	MN1764
LG03	8.7	5.9%	3.9%	0.0%	8.6%	5.4%
LG10	19.7	11.8%	17.6%	17.8%	17.3%	17.9%
LG16	11.4	0.0%	3.9%	4.4%	3.7%	9.8%

Table 4.3 Summary of FlexQTL™ runs. Two times the natural log of Bayes Factors (BF) are reported for the genome and for the likelihood of a QTL on each linkage group. BF of 2 to 5 is positive evidence, 5 to 10 is strong evidence and greater than 10 is decisive evidence of a QTL. Negative BFs do not indicate presence of QTL. Effective chain size is recommended to be about 100. n/(n-1) indicates the BF for the likelihood of n QTL opposed to n-1 QTL.

		2006 firmness	2007 firmness	2006 crispness	2007 crispness
cpu time (h:m:s)		291:55:11	286:46:14	253:34:10	236:55:21
cpu units		1.051E+09	1.032E+09	912850342	852921236
Iterations		800000	800000	700000	800000
Effective chain size of mean		215	1013	84	898
Trait summary:					
	no. samples	210	155	210	155
	mean	0.347	0.357	0.386	0.395
	variance	0.008	0.007	0.009	0.007
	minimum	0.041	0.079	0.068	0.089
	maximum	0.577	0.611	0.589	0.613
Prior assumptions:					
	no. QTL	1	1	1	1
	max. no. QTL	5	10	20	10
Results:					
2ln(BF) genome	**1/0**	NA	6.5	NA	4.8
	2/1	21.4	2.8	12.3	1.0
	3/2	7.8	1.8	3.4	0.1
	4/3	3.0	0.6	1.0	0.4
2ln(BF) LG for 1/0	**LG01**	-1.0	0.6	1.1	**2.1**
	LG02	-1.4	0.0	0.1	0.5
	LG03	**32.7**	**5.4**	**16.0**	**3.8**
	LG04	-2.2	1.6	-1.4	0.8
	LG05	-2.8	0.8	-1.6	-0.7
	LG06	0.2	0.2	-0.6	-0.6
	LG07	-0.7	1.4	**2.7**	**5.1**
	LG08	-3.0	-0.1	-1.5	-1.2
	LG09	-2.3	-0.1	-1.9	-0.5
	LG10	**18.7**	0.9	**33.1**	-0.9
	LG11	0.4	1.2	-0.8	-0.8
	LG12	-2.4	-0.2	-1.3	1.4
	LG13	-0.9	-0.5	-2.4	-0.8
	LG14	-2.2	**3.2**	-1.7	-1.4
	LG15	0.0	0.7	**2.4**	-0.9
	LG16	**10.3**	**5.9**	-0.1	1.9
	LG17	-1.8	-0.4	0.0	-1.0

Figure 4.1 Venn diagrams of number of individuals phenotyped in each year and family.

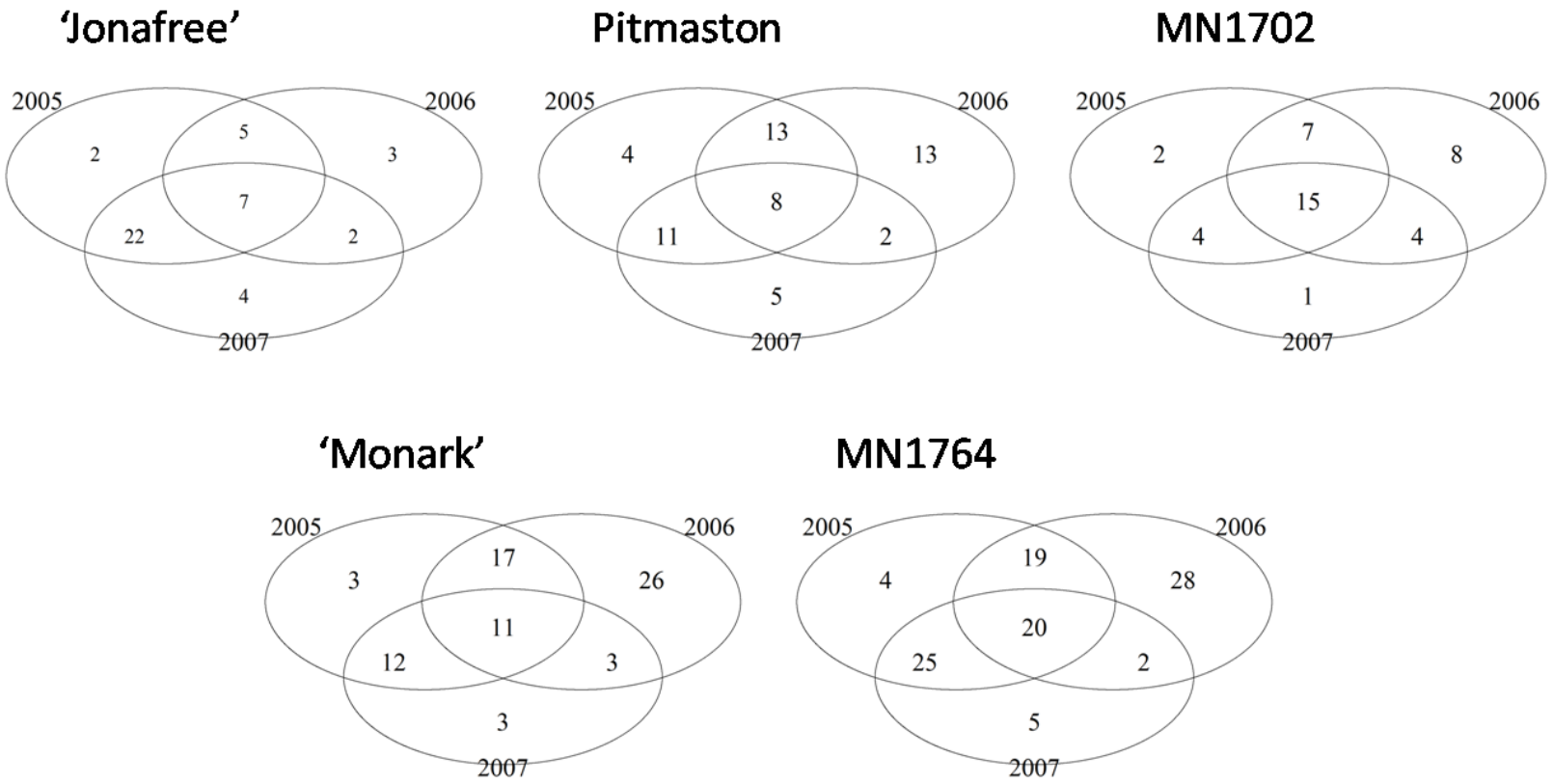


Figure 4.2 Posterior probability of quantitative trait loci positions assigned by FlexQTL™ for firmness in 2005 and 2007. Two times the natural log of Bayes Factors (BF) for the probability of one QTL on the linkage group are shown near peaks in italics. For 2ln(BF), 2 to 5 is positive evidence, 5 to 10 is strong evidence and greater than 10 is decisive evidence of a QTL.

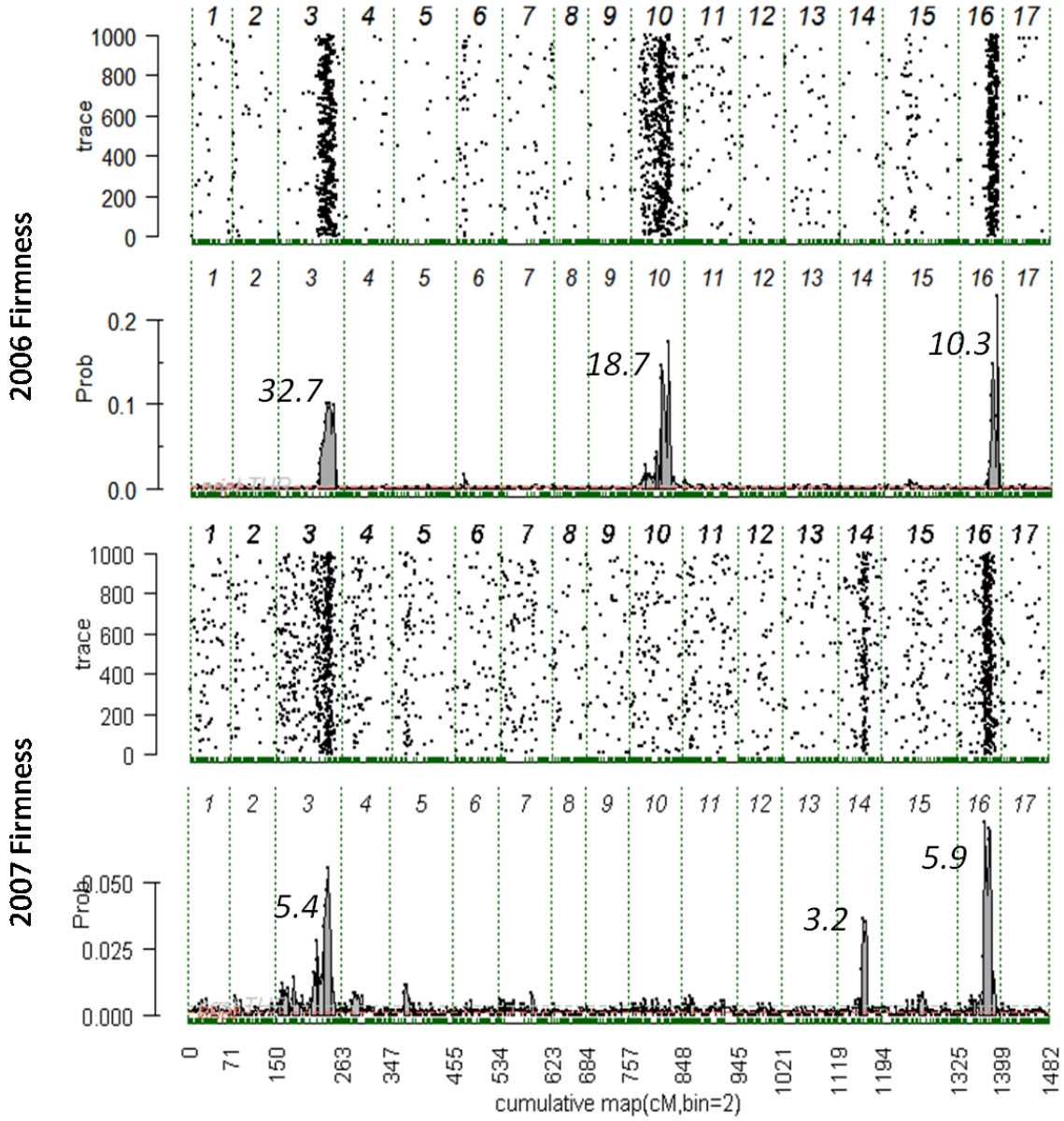


Figure 4.3 ‘Honeycrisp’ haplotypes identified on linkage groups 03, 10, and 16. Linkage group images were generated with MapChart 2.2 (Voorrips, 2002).

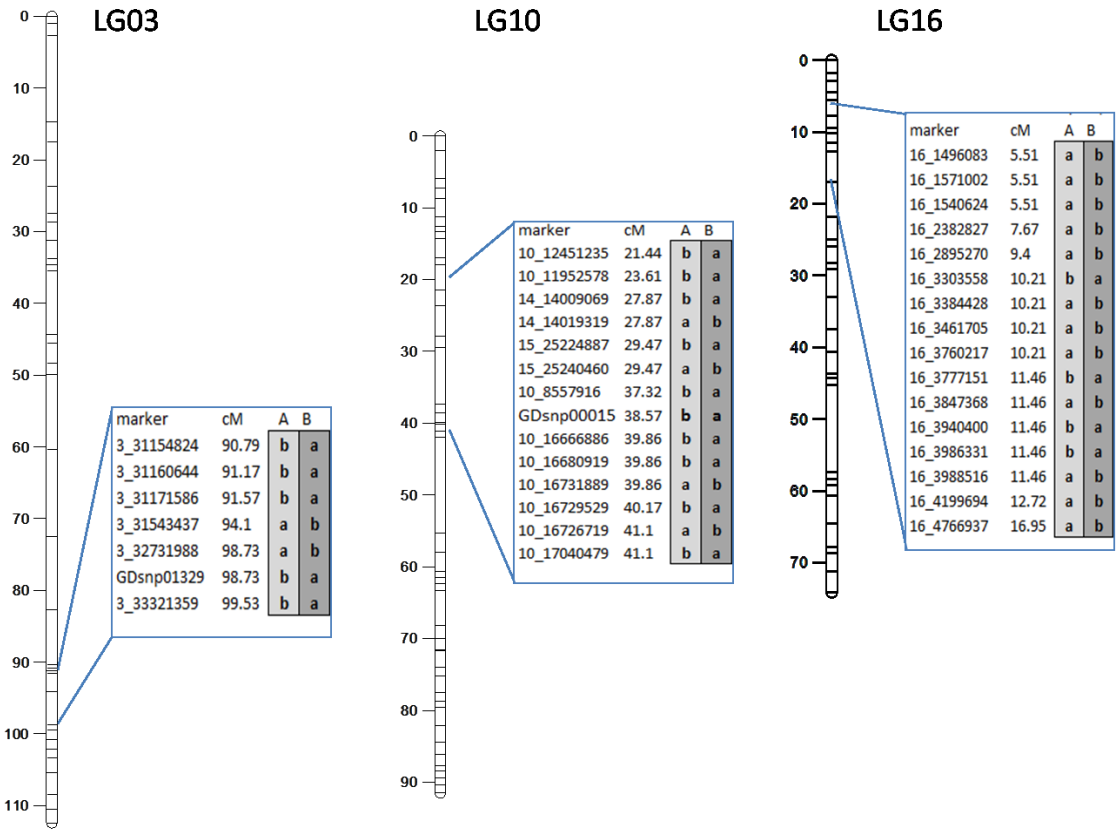


Figure 4.4 For main effects, average proportion of the total mean square (MS) attributed to each significant haplotyped region in three way ANOVAs of the traits firmness and crispness in each of three years by family. To account for unbalanced data, each ANOVA was calculated in all six possible orders and the average MS proportions for significant factors are shown, sized relative to the number of ANOVA instances in which the factor was significant at p=0.1.

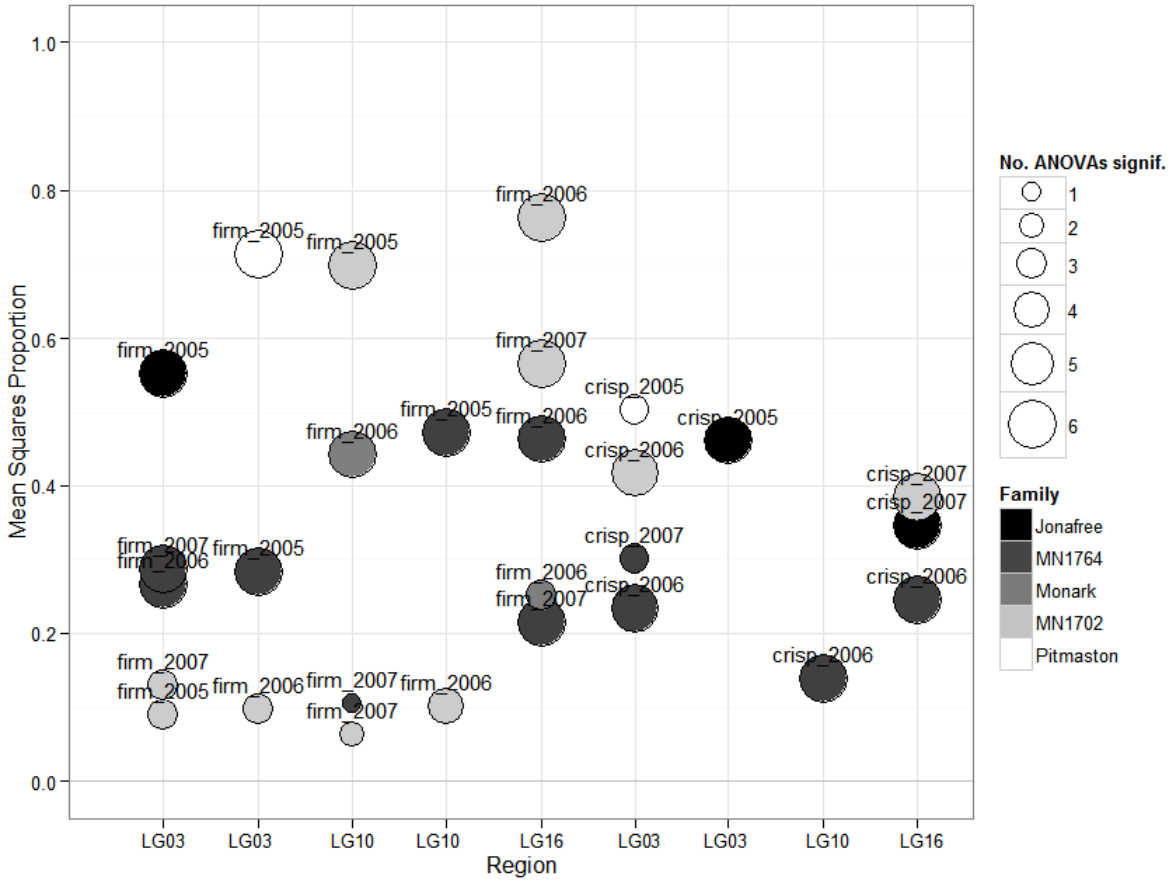


Figure 4.5 For interaction effects, average proportion of the total mean square (MS) attributed to each significant interactions of haplotyped regions in three way ANOVAs of the traits firmness and crispness in each of three years by family. To account for unbalanced data, each ANOVA was calculated in all six possible orders and the average MS proportions for significant factors are shown, sized relative to the number of ANOVA instances in which the factor was significant at the 0.1 level.

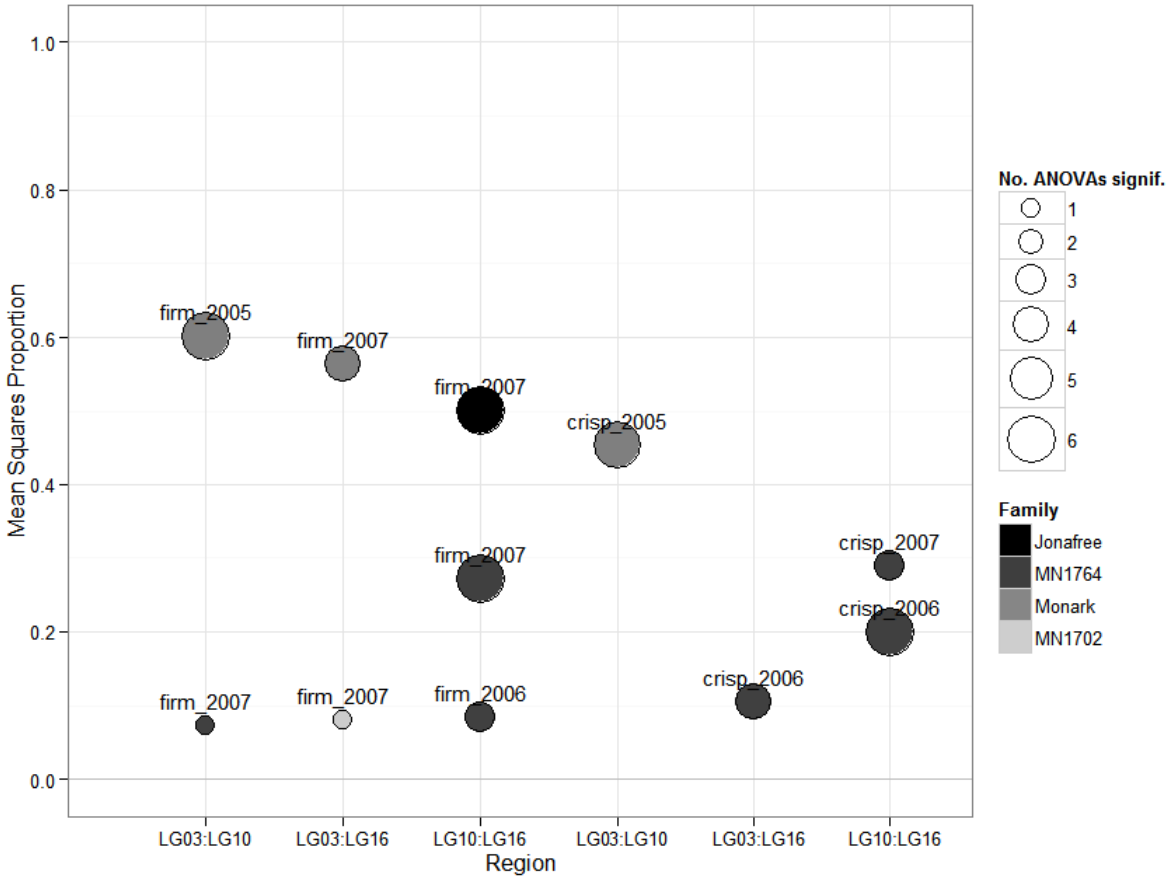


Figure 4.6 Identity by decent (IBD) probabilities for the region of interest on LG16 for 'Honeycrisp' ancestry and eight progeny of MN1702. Progeny background shade is based on 2006 firmness values where high values are darker. The progeny shown are the four highest and four lowest scoring of the family in that year. The haplotyped region is marked at either side by the strikeout of markers 16_1540624 and 16_4766937. Phenotypic data are not shown for the first three generations in this figure. The maternal homolog is always on the left. Assigned 'Honeycrisp' haplotypes are listed below the appropriate homolog.

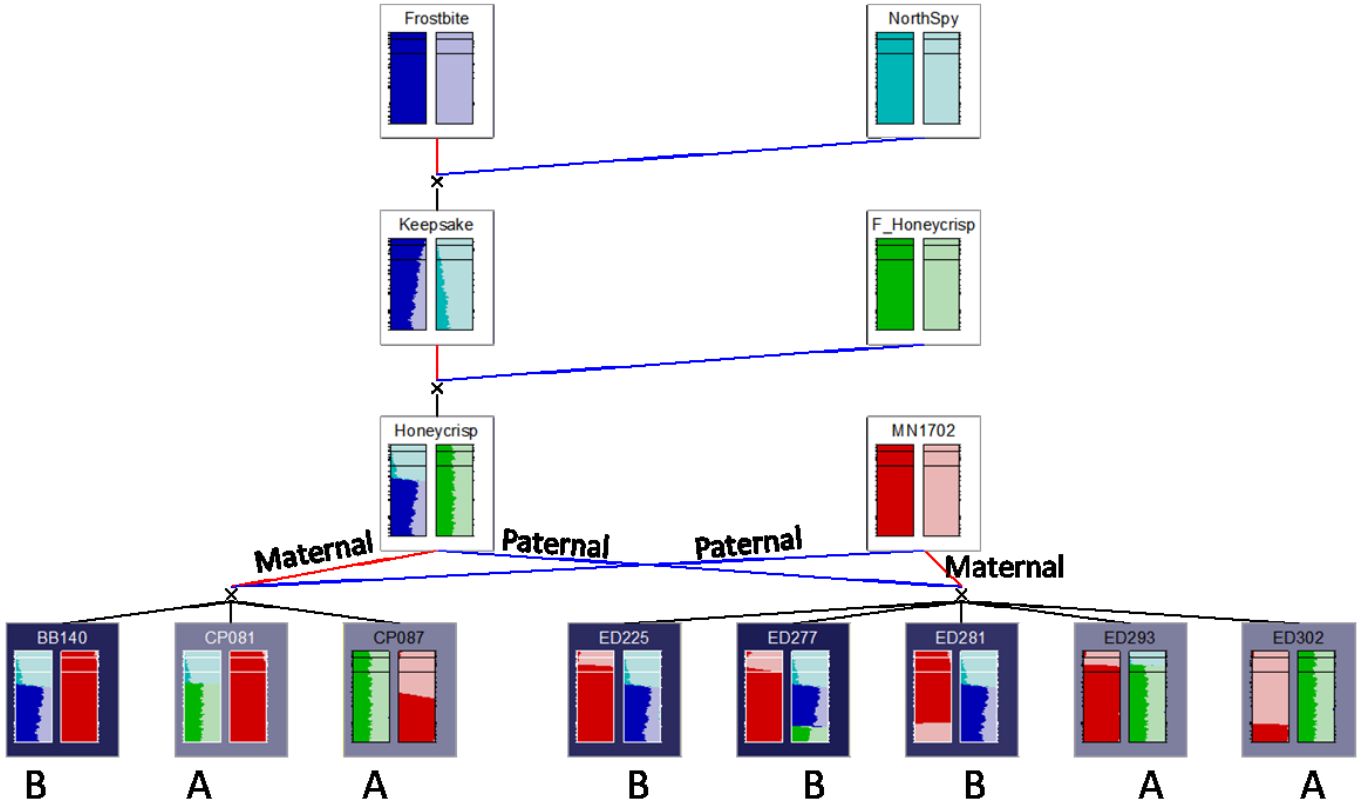


Figure 4.7 Distributions of the trait crispness, by family, of individuals having the increasing 'Honeycrisp' allele at none (0), one, two or all three of the loci (top to bottom for each family) of interest on LG03, LG10 and LG16. Narrow black vertical bars mark distribution means.

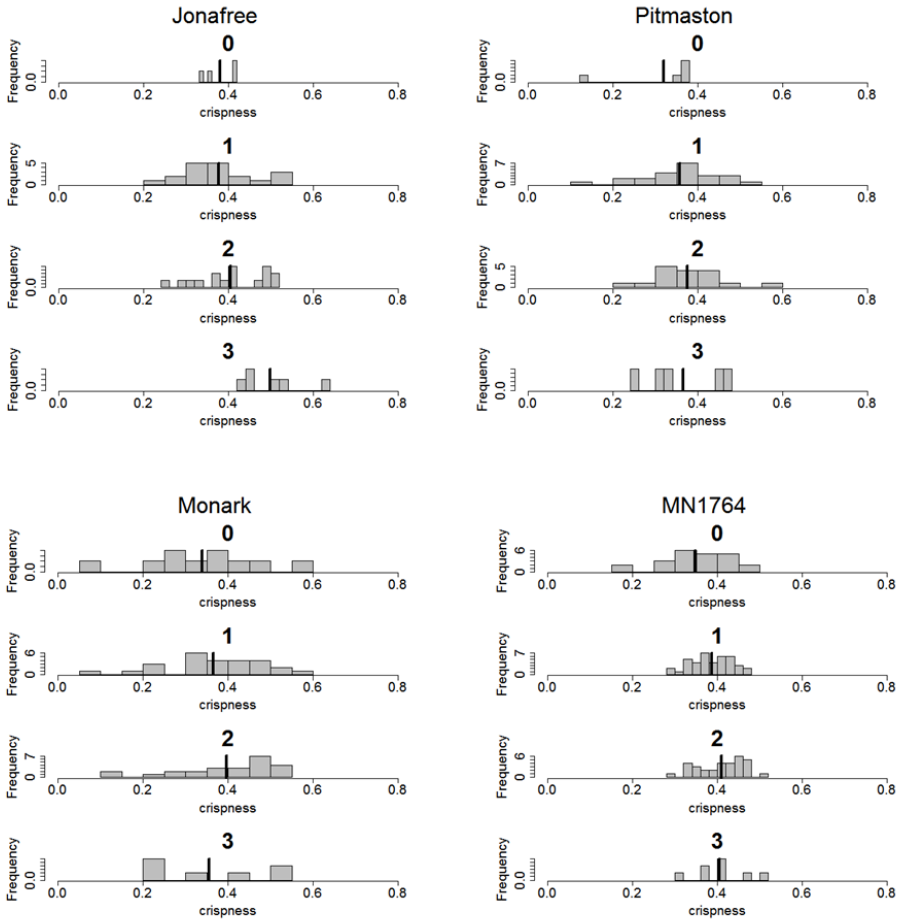
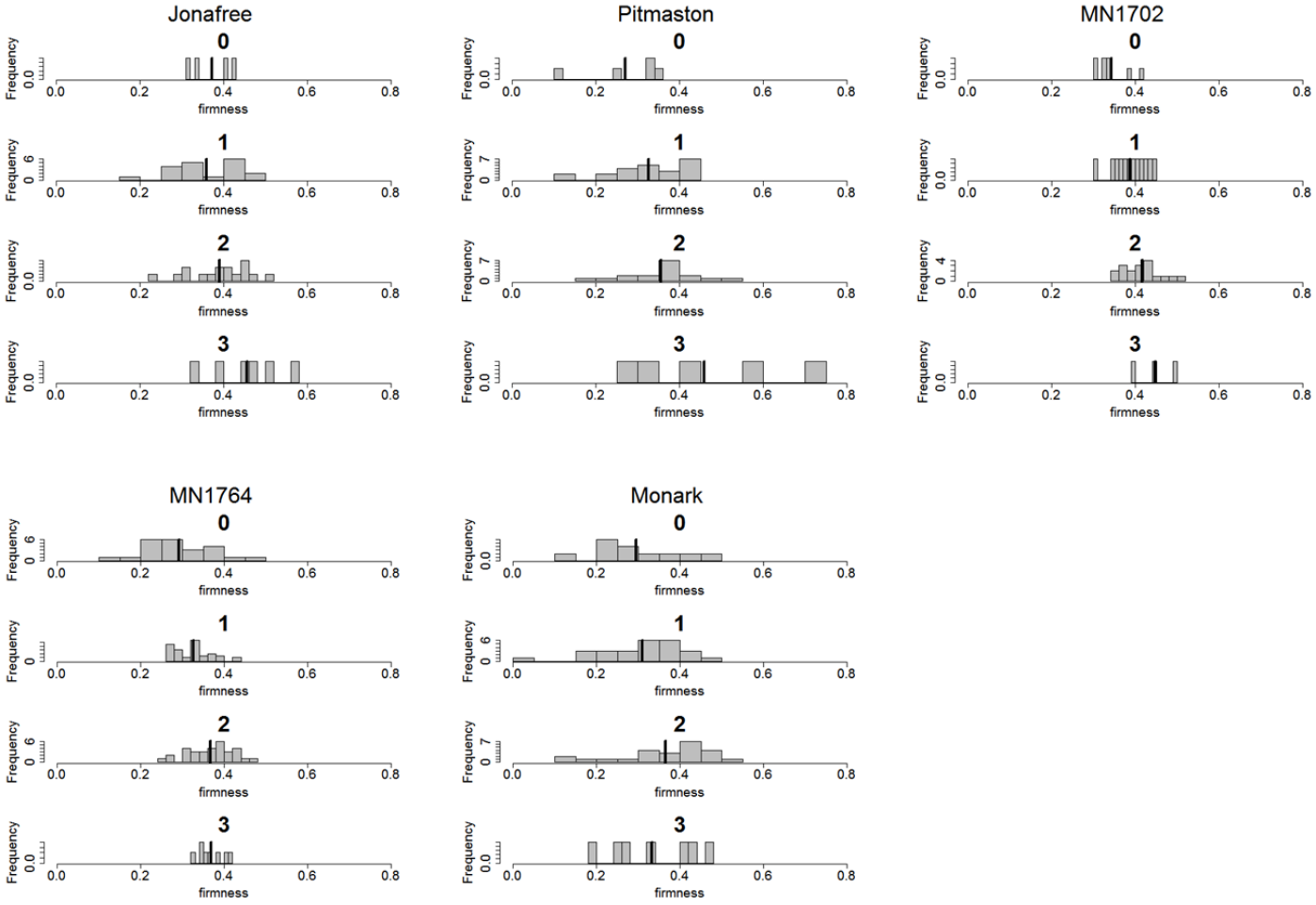


Figure 4.8 Distributions of the trait firmness, by family, of individuals having the increasing 'Honeycrisp' allele at none, one, two or all three of the loci of interest (from top to bottom) on LG03, LG10 and LG16. Narrow black vertical bars mark distribution means.



CONCLUSIONS

Establishing marker-locus-trait associations to enable marker-assisted breeding depends on having an extensive, reliable database for phenotypic traits of interest in relevant germplasm. A reference germplasm set was described in Chapter 2. The germplasm set provides efficient allelic representation of current parents in RosBREED demonstration apple breeding programs at Cornell University, Washington State University, and the University of Minnesota. The germplasm in these programs is representative of that of the United States. Within these individuals, correlations between sensory and instrumental texture measures were high in some instances, but there was no clear indication that instrumental measures could become viable replacements for sensory panels in breeding operations. Moderate year-to-year repeatability of trait values was observed, suggesting that should MAS be used to select for the texture traits studied, selections made upon the basis of marker-trait-locus associations based on a couple of years of data would likely be predictive in additional environments and across years. As each location had a largely unique set of individuals, as well as differing environmental conditions, means, ranges, and phenotypic variances differed greatly among locations for some traits. This underscores the necessity of a system of standardizing phenotypic data collection processes and the utility of implementing a process of removing year and environment effects. Non-genotypic effects could be accounted for and removed using relatedness of individuals across programs since implementing a control set common to

all sites is challenging due to winter hardiness issues imposed by the Minnesota environment.

Chapter 3 describes the use of a set of three ‘Honeycrisp’ progeny populations from the University of Minnesota apple breeding program to construct parental and consensus ‘Honeycrisp’ linkage maps. These maps are a useful tool to facilitate marker-trait association discovery that could enable marker-assisted breeding using ‘Honeycrisp’ and its descendants. Three unique ‘Honeycrisp’ parental maps were developed, among which 951 SNP markers were found to be in common. The many common markers across maps allowed these maps to be reliably merged into a consensus ‘Honeycrisp’ linkage map with 1091 SNP markers. The consensus linkage map is an informative tool for breeding programs using this elite cultivar.

QTL for fruit texture traits in ‘Honeycrisp’ progeny were presented in Chapter 4, demonstrating the use of the ‘Honeycrisp’ consensus map in identifying marker-trait-loci associations. Pedigree-based analysis was implemented to concurrently use high-quality phenotypic data from five families, which had until now only been considered independently. The use of a denser linkage map and the ability to pool all full and half sib individuals into one analysis resulted in the identification of three regions controlling crispness and firmness for which markers could be developed for MAS for texture traits. Pedigree-based analysis is cost-effective for breeding programs that maintain material over several years. It is useful in tree breeding in that it can be used to combine many sets of related materials, as often exist because the best parents are used frequently and are available year after year, as apple trees are long-lived and clonally propagated.

The techniques implemented in Chapters 3 and 4 stand as proof of concept for an application on a much larger scale. High-quality genotypic data from pedigreed individuals from across several breeding programs, like that described in Chapter 2, could be used to develop master linkage maps specific to elite cultivars or to make improvements on the arrangement of the existing apple physical map. Phenotypic data could continue to be collected with standardized protocols across breeding programs and relatedness among individuals could be used to remove environmental effects, for more accurate predictions of individual trait values. Relatedness could be further exploited to detect QTL and then define functional haplotypes through pedigree-based analysis. Meta-analysis, using data from past and present routine breeding operations across several breeding programs would highly enhance the predictive power of QTL studies, shedding more light on the nature of the loci behind quantitative traits with which population size and phenotypic data quality are often the limiting factors in the observable resolution.

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